Novel Growth Factor Complexes for Bone Tissue Engineering

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STATEMENT OF ORIGINALITY

The work contained in this thesis has not been previously submitted for a degree or diploma at any other higher education institution. To the best of this authors knowledge this thesis contains no material which has been previously published or written by another person except where due references are made.

SIGNED

DATE

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'You were not sent out to find the same results
as your predecessors but to find the truth'
Jean-Charles de Borda to Pierre-François-André Méchain
on the measurement of the French Meridian
for the determination of the length of the metre
(2nd December 1797)
(The measure of all things- Ken Alder 2002)

'The most exciting phrase to hear in science, the one that heralds new discoveries, is not 'Eureka!' (I found it!) but 'That's funny…'
-Issac Asimov

ABSTRACT

Various members of the insulin-like growth factor (IGF) family of growth factors are highly expressed in bone tissue and are vitally important for the normal development and function of bone. Recent studies have shown that IGF-I can associate with the extra-cellular matrix proteins vitronectin (VN) and fibronectin (FN) via IGF binding protein-5 (IGFBP-5). Furthermore, when these complexes are pre-bound to a tissue culture surface they can stimulate enhanced responses in epithelial cell types *in vitro*. More recently, transforming growth factor-beta 1 (TGF-β_1), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) have also been shown to interact with VN and to elicit functional responses in various cell types. Taken together, these findings indicate that exploitation of the adhesive properties of these ECM proteins might allow immobilisation of various growth factors at the culture surface. This may provide a novel means of coating engineered biomaterial constructs with agents which can elicit specific functional effects in therapeutically important cells, such as those used in cell-based therapeutics for the replacement and / or regeneration of damaged bone tissue.

Since both VN and FN are also important matrix components of bone, this study sought to investigate the hypothesis that select pre-bound combinations of these matrix proteins and growth factors could also stimulate functional responses in bone cells and the therapeutically important so called mesenchymal stem cells. Thus it is reported here that pre-bound combinations of VN, IGFBP-5 and IGF-I or FN IGFBP-5 and IGF-I significantly stimulate cell migration in the osteoblast-like SaOS-2 cells. While, VN, IGFBP-5 and IGF-I stimulated cell proliferation over 72 hr, FN, IGFBP-5 and IGF-I did not. Moreover, I found that VN, IGFBP-5 and IGF-I could facilitate alkaline phosphatase (ALP) expression in SaOS-2 cells. VN, FN and EGF on the other hand could sustain SaOS-2 cells for up to 12 days in culture, but could not sustain ALP expression; hence it is possible that these cells may have entered a state of quiescence in response to this treatment.

Extending these studies to cells derived from clinical samples, pre-bound combinations of VN / IGFBP-5 / IGF-I were not able to support initiation of human mesenchymal stem cell (hMSC) cultures. Nevertheless, VN alone in serum free

media stimulated substantial metabolic activity and protein synthesis in hMSCs once the cultures were established. Moreover, the addition of IGFBP-3 or -5 together with IGF-I can enhance the response to levels equivalent to that observed with 10% FCS. I also report that the responses to VN and TGF-β_1 are synergistic and stimulate greater hMSC metabolic activity than 10% FCS.

Interestingly, hMSCs cultured in IGF-I or TGF-β_1 and low concentrations of VN aggregated, an effect that was not observed when higher concentrations of VN were used. I hypothesise that this aggregation effect was due to endogenous protease activity, and therefore examined MMP-2 and 9 activity in hMSC conditioned media. Both pro-MMP-2 and pro-MMP-9 were constitutively expressed by hMSCs but there was no evidence of the active forms in the conditioned media, indicating that neither IGF-I nor TGF-β_1 affect MMP-2 or -9 expression or activation in serum-free media. However, hMSC conditioned media could degrade IGFBP-5, suggesting that there is proteolytic activity within the conditioned media which may impact on the function of ECM / growth factor components in serum-free media settings. Thus, while ECM and growth factors may stimulate desirable responses in therapeutically important cells in serum-free culture, the role that endogenously expressed proteases have on the efficacy of such media supplements needs to be examined closely.

Taken together, the studies reported in this thesis provide proof of principle data indicating that select combinations of ECM proteins and growth factors could be utilised in bone tissue engineering applications. This may be achieved for example, as a biomaterial coating, or could form the basis of a viable alternative media supplement for the serum-free culture of hMSCs.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABREVIATIONS

αMEM	Minimal Essential Medium-alpha formulation
ALS	Acid labile subunit
AMV	Avian myeloblastosis virus
APS	Ammonium persulphate
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
cDNA	Complementary Deoxyribonucleic acid
CIMPR	Cation independent mannose-6-phosphate receptor
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
DMEM	Dulbecco's modified Eagles medium
ECM	Extracellular matrix
EDTA	Ethylenediamine tetraacetic acid
EGF	Epidermal growth factor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FCS	Foetal calf (bovine) serum
FN	Fibronectin
HBB	HEPES binding buffer
HBSS	Hank's balanced salt solution
HEPES	4-2(2-hydroxyethyl)-1-piperazineethanesulfonic acid
IGF-I	Insulin like growth factor-I
IGF-II	Insulin like growth factor-II
IGF-IR	IGF-I Receptor
IGFBP	Insulin like growth factor-binding protein
JNK	Jun N-terminal kinase
KCl	Potassium chloride
kDa	Kilodalton
MAPK	Mitogen-activated protein kinase
$MgCL_2$	Magnesium chloride
mRNA	Messenger ribonucleic acid

MW	Molecular weight
o/n	Overnight
PAI-I	Plasminogen activator inhibitor-1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
RGD	Arginine-Glycine-Aspartate
RNA	Ribonucleic acid
RT	Room temperature
SDS	Sodium dodecyl sulphate
SDS-PAGE	SDS-Poly acrylamide gel electrophoresis
SEM	Standard error of the mean
TBE	Tris borate EDTA buffer
TE	Tris EDTA buffer
TEMED	N,N,N', N'-tetramethylethylenediamine
TGF-β_1	Transforming growth factor-beta 1
Tris	Tris[hydroxymethyl] amino methane
Triton-X100	Iso-octylphenoxypolyethoxyethanol
Tween 20	Polyethylene 20-sorbitan monolaurate
uPAR	Urokinase plasminogen activator receptor
uPA	Urokinase plasminogen activator
UV	Ultra violet
VN	Vitronectin

LIST OF PUBLICATIONS AND PRESENTATIONS

1. Schleicher I, Parker A, Leavesley D, Crawford R, Upton Z, Xiao Y: Surface modification by complexes of vitronectin and growth factors for serum-free culture of human osteoblasts. *Tissue Engineering*. 11(11/12): 1688-1698 (2005). (**Publication**)

2. Parker T, Leavesley D, Upton Z: Vitrogro Complexes in Bone Tissue Engineering. *QUT Orthopaedic Showcase* 2 Dec (2003) (**Oral presentation**)

3. Schleicher I, Parker T, Leavesley D, Crawford R, Upton Z, Xiao Y: Surface modification by complexes of vitronectin and growth factors for human osteoblast culture. *Fifth Combined Meeting of the Orthopaedic Research Societies*, Sydney, Australia, 29 Oct (2004). (**Oral presentation**)

4. Parker T, Upton Z, Leavesley D: Combinations of Vitronectin, Insulin-like Growth Factor Binding Protein-3 or-5 and Insulin like Growth Factor-I or Transforming Growth Factor-beta 1 Stimulate Proliferative Responses in SaOS-2 cells and Human Mesenchymal Stem Cells in Serum Free Conditions. *Eighth annual Tissue Engineering Society International Meeting*, Shanghai, China, 22-26 Oct (2005). (**Oral presentation**)

5. Parker T, Upton Z Leavesley D: The Functional Effects of Various Combinations of Vitronectin, IGFBP-3 or -5 and IGF-I or TGF-β_1 on SaOS-2 cells and Human Mesenchymal Stem Cells in Serum Free Conditions. *Third International Congress of the Growth hormone Research Society and the Insulin-Like Growth Factor Society*, Kobe, Japan, Nov (2006). (**Oral presentation**)

6. Parker T, Whiteside E, Yameen Y, Upton Z, Leavesley D: Complexes of IGFs, IGFBPs and Vitronectin Stimulate Proliferation and MMP Secretion by Osteoblast-Like SaOS-2 Cells. *Second International GH-IGF Symposium*, Cairns, Australia, 18-22 Apr (2004). (**Poster presentation**)

7. Parker T, Whiteside E, Yameen Z, Upton Z, Leavesley D: Complexes of IGFs, IGFBPs and Vitronectin Stimulate Proliferation and MMP Secretion by Osteoblast-Like SaOS-2 Cells. *Australian Society of Medical Research Student Conference*, Wesley Hospital, Brisbane, Australia, Jun (2004). (**Poster presentation**)

8. Parker T, Yameen Z, Upton Z, Leavesley D: Combinations of Vitronectin, Insulin like Growth Factor Binding Protein 3 or 5 and Insulin like Growth Factor-I stimulates initial proliferative responses in SaOS-2 cells and hMSCs in serum free conditions. *Australian Society of Medical Research Student Conference*, Wesley Hospital, Brisbane, Australia, Jun (2005). (**Poster presentation**)

9. Parker T, Upton Z Leavesley D: The Functional Effects of Various Combinations of Vitronectin, IGFBP-3 or -5 and IGF-I or TGF-β_1 on SaOS-2 cells and Human Mesenchymal Stem Cells in Serum Free Conditions. *Australian Society of Medical Research Student Conference*, Wesley Hospital, Brisbane, Australia, Jun (2006). (**Abstract**)

10. **Parker T**, Upton Z, Leavesley D: The Functional Effects of Vitronectin or Fibronectin, IGFBP-5 and IGF-I Complexes on SaOS-2 cell Migration and Proliferation in Serum Free Conditions. *IGF's Down Under-Bound Up All Over*, Brisbane, Sept 29 (2006). (**Poster presentation**)

11. **Parker T**, Upton Z, Leavesley D: The Functional Effects of Vitronectin or Fibronectin, IGFBP-5 and IGF-I Complexes on SaOS-2 cell Migration and Proliferation in Serum Free Conditions. *Combio 2006* Brisbane Sept 24-28 (2006). (**Poster presentation**)

CHAPTER 1:

LITERATURE REVIEW

1.1 INTRODUCTION

The term ‘tissue engineering’ was coined by members of a panel representing the National Science Foundation (NSF), Bioengineering and Research to Aid the Handicapped (BRAH) and Biotechnology (BIOTECH) who gathered to discuss future directions in bioengineering in the United States. While, BIOTECH desired novel methods for homologous cell culture, BRAH sought the development of whole organ replacement systems. The two research areas appeared to overlap, and in order to consolidate their efforts the term ‘tissue engineering’ was developed. The term was formally defined at a workshop to identify appropriate areas for engineering research in the new emerging technology of tissue engineering, held at Lake Tahoe, California on February 26-29, 1988. The first research proposals were received and funded in 1988. Over recent decades tissue engineering has become a major global research area in biotechnology (Patrick *et al.* 1998).

Broadly defined ‘tissue engineering’ is the use of living cells together with either natural or synthetic extra-cellular components *in vitro*, for the development of implantable parts or devices for the restoration or replacement of tissue or organ function (Patrick *et al.* 1998). In order to regenerate living tissue *in vitro*, cells must be organised and behave as if they are part of the original tissue *in vivo*. In layman’s terms this means that the cells must ‘sense’ they are in their natural environment. Cells must be able to proliferate, differentiate and/or migrate and this then implies they must receive, from their new environment, the appropriate cues, which facilitate and direct these processes. A critical component of appropriately organising cells and their functions into tissues, is their association with the extra-cellular matrix (ECM) (Ross 1998). The ECM is a structural material made by the cells which acts as a scaffold within which the cells reside and is largely composed of tissue specific mixtures of glycoproteins and proteoglycans. Cells interact directly with the ECM via specific cell surface receptors, which are often associated with intra-cellular signalling networks; thus cell / ECM interactions can induce signalling events within the cells and thereby modify cell function. Local variation in the composition of the ECM can result in temporal and spatial variation in cellular responses and thus tissue structure and function. A volumous literature has been produced over the last decade describing the association of growth factors and cytokines with various ECM

components and mediation of cell function through cell / ECM interactions (Jones *et al.* 1993; Slater *et al.* 1995; Taipale *et al.* 1998; Nam *et al.* 2000; Conover and Khosla 2003; Hutchings *et al.* 2003; Russo *et al.* 2005). Significant among these are the discoveries that many of the components of the insulin-like growth factor (IGF) family of growth factors and associated proteins are observed to associate with vitronectin (VN)(Upton *et al.* 1999; Nam *et al.* 2002; Kricker *et al.* 2003), a ubiquitous 75 kDa ECM glycoprotein (Schvartz *et al.* 1999). Since this initial observation epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) and transforming growth factor-β (TGF-β) have also been shown to associate with VN (Schoppet *et al.* 2002; Hollier *et al.* 2005). Notwithstanding, VN is also known to facilitate integrin and urokinase system mediated cell attachment (Ruoslahti and Pierschbacher 1987; Leavesley *et al.* 1992; Felding-Habermann and Cheresh 1993; Kanse *et al.* 1996).

One of the most active areas of research within tissue engineering is the field of 'Bone tissue engineering'. Investigators from the traditional disciplines of biomedical and chemical engineering, polymer chemistry, biochemistry, cell and molecular biology, genomics and physiology conduct research in this field. Likely due to this cross disciplinary approach to research, a huge volume of literature has been generated in the last decade regarding the incorporation and convergence of ideas between these disciplines for the purposes of developing better bone therapeutics for the treatment of musculoskeletal pathologies. For example, production of biomaterials such as hydroxyapatite by materials chemists and engineers are now being investigated by cell and molecular biologists for their effects on cell behaviour. Furthermore, incorporation of various ECM components with these biomaterials is also being investigated to modify cell behaviour through their combined effects.

The last decade has also seen the emergence of methodologies enabling isolation and culture of human mesenchymal stem cells (hMSC). hMSCs have a demonstrated potential to generate multiple tissue types including bone and cartilage. Many modern strategies for the development of advanced connective tissue therapeutics are focusing on implantable constructs consisting of a biomaterial scaffold, incorporating some form of ECM and / or *ex vivo* expanded, undifferentiated

hMSCs. The underlying concept is to deliver a large number of precursor cells, which subsequently differentiate into appropriate mature tissue types through guiding interactions with both the accompanying scaffold / ECM and the surrounding host tissue.

Interactions between the ECM and components of the IGF system and other biologically active factors such as TGF-β are believed to have wide ranging effects on bone tissue growth and turnover, thus facilitating increased interest in the potential use of these compounds in the development of novel therapeutics for treatment of a variety of bone diseases. Likewise there is increasing interest in the incorporation of hMSCs into novel tissue engineered therapeutics. With this background in mind, in addition to providing a brief background on bone, bone remodelling and discussion of the current literature on hMSCs, this review will concentrate on discussion of the components of the IGF system, TGF-β_1 and VN. In particular the review will be focussed on their interactions and effects on hMSCs, localisation in bone and role in bone remodelling.

1.2 BONE STRUCTURE AND FUNCTION: OVERVIEW

Bone is a specialised form of connective tissue in which its extra-cellular components are mineralised, conferring both strength and a degree of elasticity with a minimum of bony mass. In addition to structural support for the body as a whole, bone also provides protection to soft organs such as brain, heart and lungs etc and facilitates movement due to the particular arrangement of bones, joints, ligaments, tendons and skeletal muscle tissue. Bone, playing a vital role in calcium homeostasis, serves as a repository for calcium and other inorganic ions and provides the site of hematopoiesis within the marrow cavity of some bones (Heaney 2003; Aguila and Rowe 2005).

Macroscopically compact bone appears solid and dense. However, microscopically compact bone contains a mass of canals that serve as conduits for blood vessels, lymphatic vessels and nerves. The basic structural unit of compact bone is called an osteon, which consists of concentric rings of bone matrix lamellae, arranged around a central conduit named the Haversian canal (Burkitt *et al.* 1993). Type 1 collagen is

the major fibrous constituent of bone and is arranged in a common direction in each lamella but in opposing directions in adjacent lamellae (Burkitt *et al.* 1993; Sommerfeldt and Rubin 2001; Locke 2004). The central cavity of bone consists of a meshwork of porus bone called cancellous or trabecular bone. Thus, bone is both lightweight and strong due to the combination of the strength characteristics of the haversian system and lamellar bone and the support characteristics of cancellous bone (Figure 1.1).

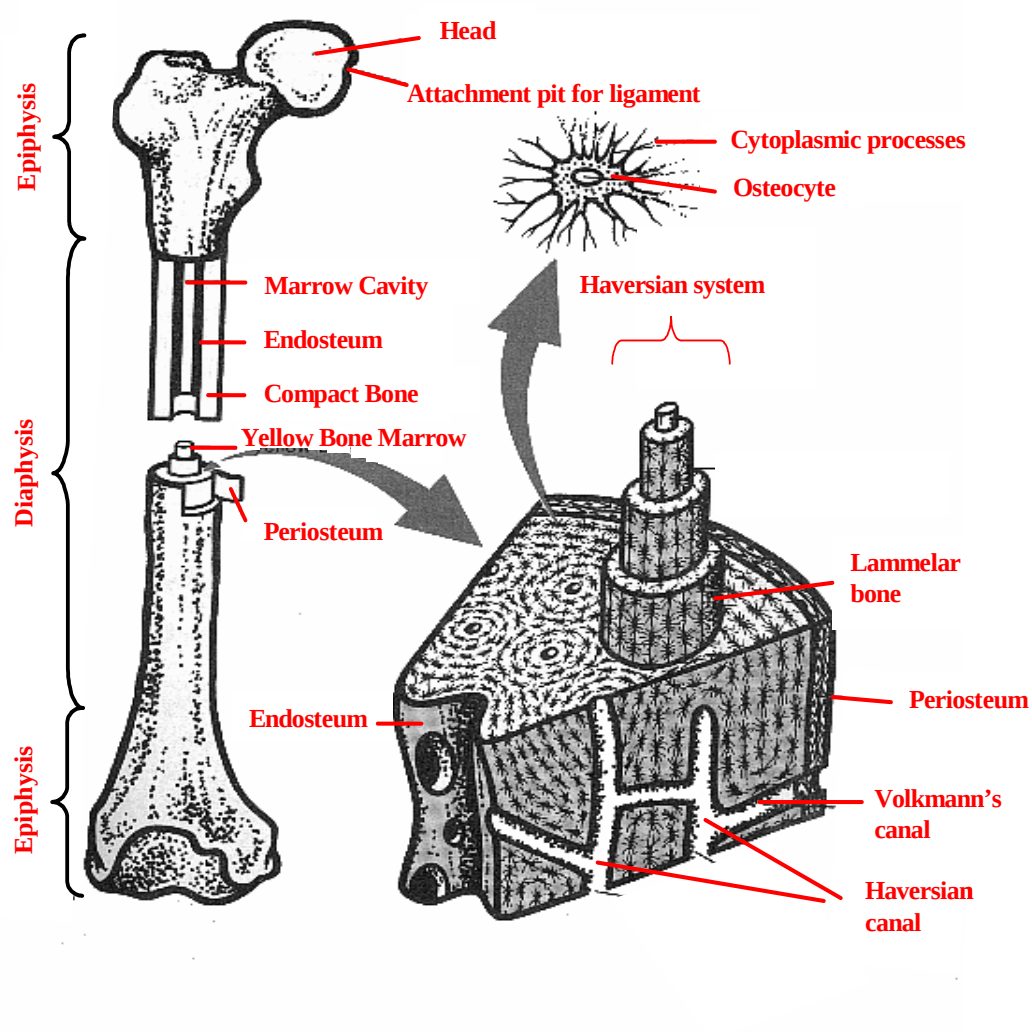


Figure 1.1 Schematic diagram of the gross and microscopic structure of long bones. (www.botany.uwc.ac.za/sci_ed/ grade10/mammal/bone.htm)

1.2.1 Bone remodelling: Overview

Bone remodelling is performed by osteoclasts and osteoblasts; together these cells form a functional unit called the bone metabolic unit (BMU), also sometimes called

the basic multi-cellular unit (Sommerfeldt and Rubin 2001). The BMU is responsible for the maintenance of calcium and phosphate homeostasis in addition to bone growth and maintenance of bone integrity. This is achieved through constant bone resorption and synthesis of new bone. Osteoclasts resorb bone while osteoblasts synthesise new bone matrix (osteoid) and provide the enzymes that facilitate the process of matrix mineralisation with calcium and phosphate ions to form hydroxyapatite (HA) ($Ca_{10}(PO_4)_6$).

1.2.2 Osteoclasts

Osteoclasts are large multinucleated cells that are derived from hematopoietic stem cells and are responsible for bone resorption (Suda *et al.* 1992). The sequence of cellular events involved in bone resorption is referred to as the resorption cycle. The osteoclast-mediated resorption cycle includes: migration of the osteoclast to the resorption site; attachment of the osteoclast to the bone surface; polarisation and formation of new osteoclast plasma membrane domains; dissolution of hydroxyapatite, degradation of the organic matrix; removal of degradation products from the resorption lacuna; and finally, either return to a non-resorbing phenotype or the osteoclast undergoes apoptosis (Vaananen *et al.* 2000). Morphologically, the most characteristic feature of osteoclasts is the presence of a 'ruffled border', a highly convoluted region of the plasma membrane adjacent to the bone surface. The adjacent cytoplasm contains a large number of lysosomes, which are involved in the degradation of resorbed bone matrix (Figure 1.2). Surrounding the ruffled border is a region known as 'the sealing zone', where the plasma membrane is relatively uniform. Actin filaments are concentrated within the cytoplasm of the sealing zone and are surrounded by a ring of cytoskeletal and adhesion molecules such as vinculin and talin. Interestingly, some authors have suggested that the sealing zone membrane contains numerous VN receptors ($\alpha_v\beta_3$ integrins) and that osteoclast attachment is mediated by sealing zone $\alpha_v\beta_3$ integrins binding to a variety of ECM proteins including VN (Davies *et al.* 1989) and osteopontin (Reinholt *et al.* 1990). More recently, however, others have failed to demonstrate $\alpha_v\beta_3$ localisation in the sealing zone (Lakkakorpi *et al.* 1991; Masarachia *et al.* 1998; Duong *et al.* 2000) although it was found to be important for proper osteoclast function. Still others have suggested some members of the cadherin family are important for the tight attachment of the

sealing zone to the bone surface (Ilvesaro *et al.* 1998). Thus, the specific molecules responsible for mediating osteoclast sealing zone attachment to the bone surface remain to be identified unequivocally. The resorption area under the ruffled border, called the Howship's lacuna, is acidic, which favours dissolution of bone mineral (Baron *et al.* 1985). This is due to vacuolar type H+-ATPase proton pump, located in the plasma membrane of the ruffled border, which transport protons into the resorption lacuna, thereby decreasing the pH of the microenvironment (Vaananen *et al.* 1990) (Figure 1.2).

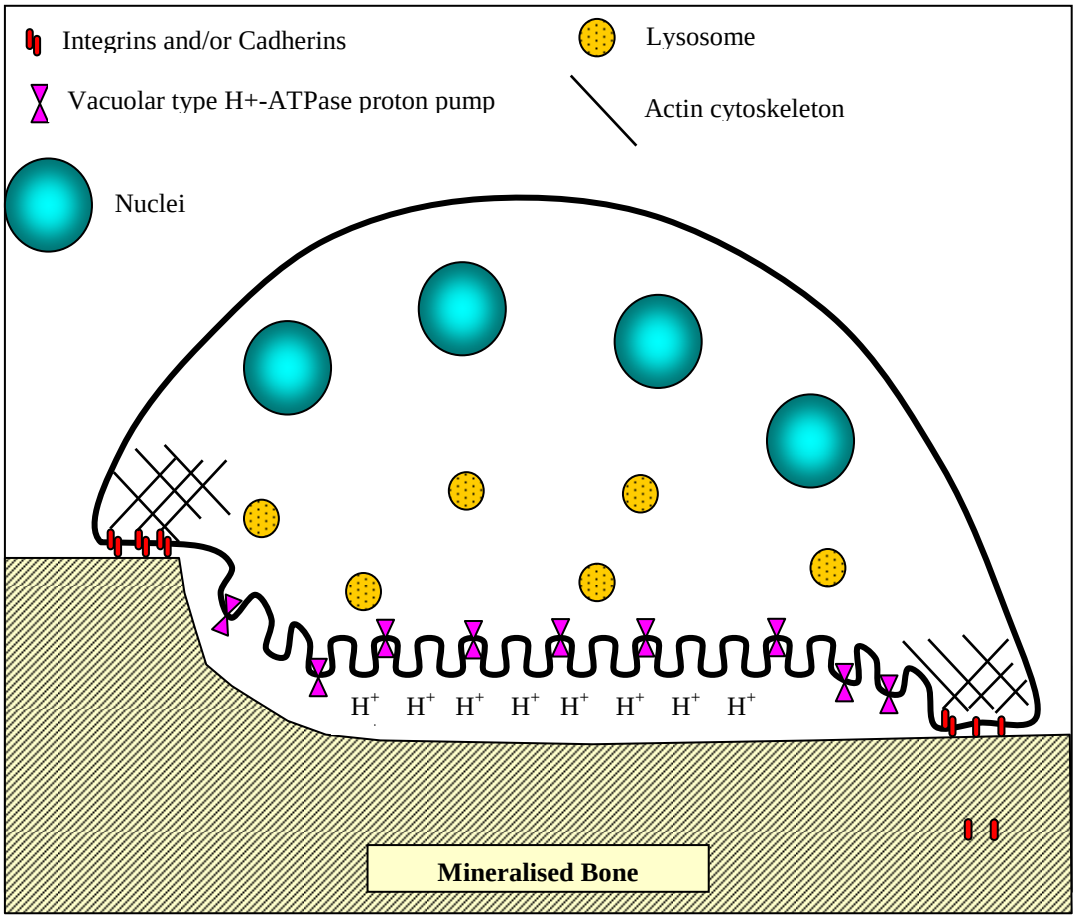


Figure 1.2. **Schematic diagram of osteoclast structure and microenvironment.** Osteoclasts are giant multinucleate cells of the hematopoietic lineage that are responsible for the resorption of bone. Resorption of the mineral component of bone (hydroxyapatite) is facilitated by the production of an acidic microenvironment beneath the osteoclast via the action of vacuolar type H^+-ATPase proton pumps located in the osteoclast ruffled border. Cell attachment molecules such as integrins and / or cadherins mediate osteoclast attachment to the bone surface through interactions with select bone ECM proteins and to the intracellular actin cytoskeleton through cytoskeletal adhesion complexes such as vinculin and talin.

1.2.3 Osteoblasts

Osteoblasts are mononucleate cells of mesenchymal origin, which produce bone ECM and enzymes that facilitate the matrix mineralisation processes. Both hormonal and non-hormonal molecules stimulate proliferation and differentiation processes in osteoblasts. These include, but are not limited to, thyroid hormone (TH) (Pepene *et al.* 2001), parathyroid hormone (PTH) (McCarthy *et al.* 1989b), growth hormone (GH), prostaglandins (Hakeda *et al.* 1991), oestradiol (Ernst *et al.* 1989; Westley and May 1994), TGF-β, IGF-I and IGF-II (Hock *et al.* 1988; McCarthy *et al.* 1989a; Yakar *et al.* 2002). The differentiation process of the osteoblast lineage is well defined and is characterised by coordinated progressive changes in cell morphology accomplished by transient expression of various bone specific and bone non-specific proteins, bone matrix formation and matrix mineralisation. Terminally differentiated osteoblasts undergo apoptosis, become bone-lining cells (senescent osteoblasts), or become embedded in the matrix and become osteocytes, which survive in small spaces between the lamellar bone known as lacunae. Osteocytes are thought to form a continuous syncytium that can sense mechanical loads acting on bone and monitor bone integrity and allow communication with the bone lining cells. Thus this large network of interconnected cells has the potential to orchestrate the spatial and temporal recruitment of cells of the BMU (Sommerfeldt and Rubin 2001). This is achieved through cell / cell interactions between osteocytes via specialised cytoplasmic processes, which exist in small channels called canaliculi and link individual lacunae to each other and to the central haversian canal system.

Transient expression of ECM proteins by osteoblasts results in the formation of new bone matrix into which secreted growth factors such as the IGFs, IGF-binding proteins (IGFBPs), and TGF-βs (which include the bone morphogenetic proteins (BMPs)) become incorporated. These are stored in the matrix and upon release during bone turnover / remodelling events can alter the behaviour of subsequent generations of osteoblasts in a paracrine manner (Mundy *et al.* 1995). This further suggests that an age-related down regulation of these genes, and subsequent reduced concentration of the corresponding proteins, may partially explain the reduced osteoblast activity observed in pathological conditions such as osteoporosis (Mohan *et al.* 1995a).

1.2.4 Human Mesenchymal Stem Cells

Human osteoblasts arise from human mesenchymal stem cells (hMSCs) which most commonly reside within the bone marrow. hMSCs are thought to be the cells which facilitate bone remodelling and repair processes (Bruder *et al.* 1998a; Richards *et al.* 1999). hMSCs are characterised as being multipotential, that is they are able to differentiate variously into cartilaginous tissue (Johnstone *et al.* 1998; Pittenger *et al.* 1999; Majumdar *et al.* 2000), bone tissue (Bruder *et al.* 1997b; Jaiswal *et al.* 1997; Pittenger *et al.* 1999; Lennon *et al.* 2000), tendinous tissue (Young *et al.* 1998), muscle tissue (Wakitani *et al.* 1995), adipose tissue (Pittenger *et al.* 1999) and stromal tissue which supports haematopoiesis (Majumdar *et al.* 1998), while also being capable of self renewal (Caplan 1994) (Figure 1.3). Cultured hMSCs have a demonstrated ability to be passaged and expanded up to 25 times without significant changes in morphology, growth pattern or immunophenotype. This translates into an approximate 550 million fold increase in cell number (Conget and Minguell 1999). These authors also demonstrated that hMSCs cultured and expanded in the absence of differentiation stimuli express cell surface antigens associated with mesenchymal, endothelial and epithelial cell lineages (Conget and Minguell 1999).

MSC differentiation into a given tissue type, for example bone, is regulated by specific growth factors, cytokines, vitamins and microenvironment / ECM to which the hMSC is exposed (Bruder *et al.* 1997b; Jaiswal *et al.* 1997; Pittenger *et al.* 1999; Coelho *et al.* 2000; Coelho and Fernandes 2000; Lennon *et al.* 2000; Bennett *et al.* 2001a). hMSCs have also been shown to be involved in osteoclastogenesis *in vitro*. Experimental co-cultures of human haematopoietic stem cells (hHSCs) and human MSCs (hMSCs) indicated that terminal differentiation of hHSCs into osteoclasts (Ocls) is dependent on cell-to-cell contact and the provision by hMSCs of osteoclastogenic factors to the hHSCs (Mbalaviele *et al.* 1999). Interestingly, these data further suggested that hHSCs induce hMSCs to produce cytokines that regulate osteoclastogenesis. These findings also suggest that while hMSCs constitute the source of new tissue for purposes such as wound repair, they are also important regulators of homeostatic mechanisms such as integration and control of the BMU.

In vitro, expanded MSCs have been shown to synthesise bone when seeded onto biodegradable collagen scaffolds and implanted into large segmental bone defects in

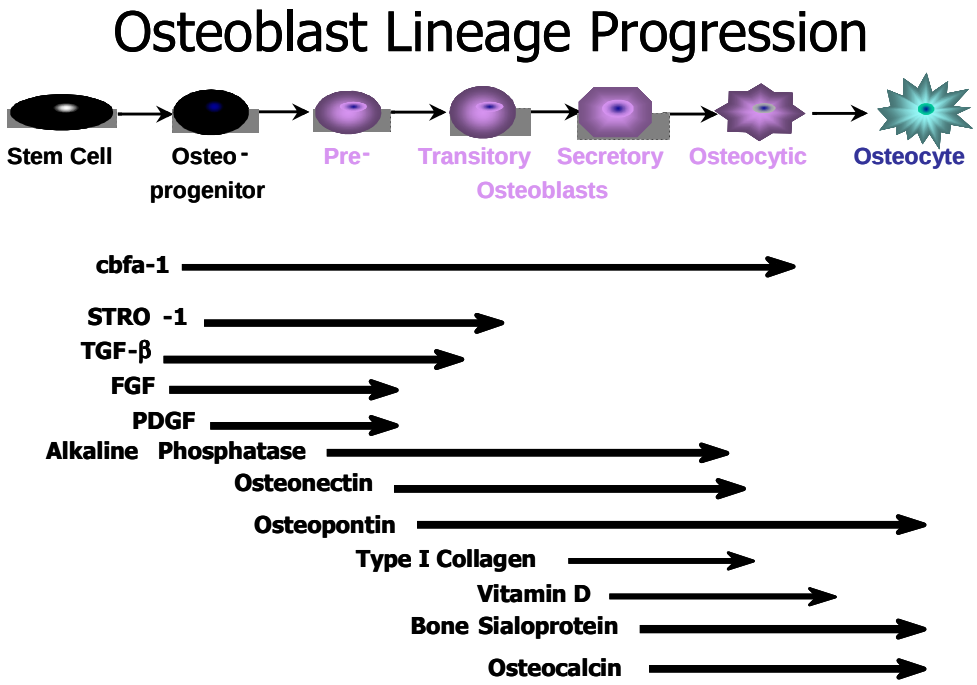


Figure 1.3. **Specific osteoblast lineage markers**. SH-2, SB-10, SB-2. SB-3, SB-5 and STRO-1 are monoclonal antibodies that recognise specific cell surface antigens on the respective cells of the osteoblast lineage. Also shown are the temporal expression profiles of Transforming Growth Factor-β (TGF-β), Fibroblast Growth Factor (FGF), Platelet Derived Growth Factor (PDGF), Alkaline Phosphatase (ALP), Osteonectin, Osteopontin, Type 1 Collagen (Col-1), 1,25-dihydroxyvitamin D3 (Vitamin D), Bone Sialoprotein and Osteocalcin mRNA. Adapted from (Bruder *et al.* 1997a).

mice (Turgeman *et al.* 2001). MSCs seeded onto hydroxyapatite / β-tricalcium phosphate scaffolds can support the regeneration of bone when implanted into a significant clinical defect in the femurs of rats. The newly synthesised bone tissue exhibits superior biomechanical and histomorphometrical properties to the implanted hydroxyapatite / β-tricalcium phosphate scaffolds alone (Bruder *et al.* 1998b). Thus hMSC-seeded scaffolds have a demonstrated ability to repair bone defects in animal models and this strongly suggests that this technology offers an equivalent potential in human patients. Importantly, the proliferative capacity and population of hMSCs are similar in osteoporotic patients to healthy individuals, irrespective of age (Stenderup *et al.* 2001). These data support our view that hMSCs harvested from patients own bone marrow, expanded in culture and seeded onto orthopaedic implants coated with osteoinductive matrix / growth factors will enhance osseointegration of the implant.

Of the various cell lineages into which hMSCs are capable of differentiating, the osteoblastic lineage is the most thoroughly understood. hMSC osteoblastic differentiation has been characterised (Figure 1.3) using various antibodies to specific cell surface antigens, enzyme activity assays, mRNA expression and mineralisation (Haynesworth *et al.* 1992a; Haynesworth *et al.* 1992b; Bruder *et al.* 1997a; Jaiswal *et al.* 1997; Oreffo *et al.* 1999; Yamaguchi *et al.* 2000; Bennett *et al.* 2001a; Kawaguchi *et al.* 2001; Papergerakis *et al.* 2002). Transiently expressed proteins have been used extensively to define various intermediate stages of cells differentiating along the osteoblast lineage. Thus, determination of the protein / antigen expression profile of cells cultured on a particular biomaterial or ECM / growth factor complex is indicative of the potential of the substrate for the support of differentiation of cells along the osteoblastic lineage. The capacity of cells to differentiate appropriately while attached to a surface is an important consideration for both *ex vivo* and *in vivo* bone healing and regeneration processes. I envisage that this will help facilitate osseointegration of biomaterials with existing bone tissues.

1.2.5 Osteoblast-like SaOS-2 Cells

Although, originally isolated from an osteosarcoma from an 11 year old female in 1977 and thus having potential for abnormal gene expression, SaOS-2 cells have become a popular cell line model for developmental studies of osteoblast responses to various treatments. This popularity is partly due to their clonal expansion ability which provides large homogeneous populations of osteoblast-like cells (Murray *et al.* 1987; Farley *et al.* 1991; Farley *et al.* 1993; Okumura *et al.* 2001; Kilpadi *et al.* 2004). The osteoblast-like properties of SaOS-2 cells were initially described by Rodan and co-workers in 1987 (Rodan *et al.* 1987). These properties include: production of a mineralised matrix; parathyroid hormone-sensitive adenylate cyclase activity; osteonectin production and secretion; presence of 1,25-dihydroxyvitamin D_3 receptors; and significantly, cell density dependent-alkaline phosphatase activity, (Rodan *et al.* 1987). Of importance to the research described in this thesis, SaOS-2 cells express $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, classical cell surface receptors for VN and FN respectively (Koistinen *et al.* 1999; Postiglione *et al.* 2003; Kilpadi *et al.* 2004). SaOS-2 cells also express IGF-I receptors and are responsive to IGF-I (Bilbe *et al.* 1996; Nasu *et al.* 2000; Grey *et al.* 2003; MacEwen *et al.* 2004). In addition, they have been shown to express epidermal growth factor receptor (EGFR), fibroblast

growth factor-I receptor (FGF-IR) and type I, type II and III (betaglycan) TGF-β receptors (Takeuchi *et al.* 1995; Bilbe *et al.* 1996). Thus SaOS-2 cells are a useful model cell line model for evaluating osteoblastic responsiveness to matrix proteins such as VN and FN and to growth factors such as IGF-I, EGF, and TGF-β_1.

1.3 INTEGRINS

Integrins are cell surface transmembrane glycoproteins of a heterodimeric structure of non-covalently bound α and β subunits and mediate cellular attachment to the ECM (Pierschbacher and Ruoslahti 1984). Various combinations of the 16 known α and 8 β subunits form 22 distinct heterodimers, with each determining ligand-binding specificity. Integrins, including those expressed by bone cells (osteoclasts and osteoblasts), consist of a large extra cellular domain, a single hydrophobic transmembrane domain and a short cytoplasmic domain. The extra-cellular domain has been shown to be responsible for binding / interacting with ECM proteins through highly specific peptide recognition motifs such as the Gly-Glu-Arg (GER) sequence of type 1 collagen (Knight *et al.* 1998), recognised by $\alpha_2\beta_1$ and the Arg-Gly-Asp (RGD) sequence present in many ECM proteins, including fibronectin (Pierschbacher and Ruoslahti 1984), osteopontin (Reinholt *et al.* 1990), bone sialoprotein (Byzova *et al.* 2000) and of particular interest to this author, VN (Pytela *et al.* 1985) and recognised by $\alpha_v\beta_3$ integrins.

In addition to cell adhesion, ligation of integrin receptors stimulates intracellular signalling events such as tyrosine phosphorylation (Schwartz *et al.* 1995) and activation of focal adhesion kinase (FAK), as well as mitogen-activated protein (MAP) kinases (Chen *et al.* 1994) such as extra-cellular signal-related kinase (ERK). Interestingly, integrin ligand occupancy and aggregation have been shown to induce EGF, PDGF and bFGF receptor accumulation at sites of integrin aggregation, resulting in enhanced growth factor receptor tyrosine phosphorylation and enhanced downstream ERK activity (Miyamoto *et al.* 1996). Lai et al (2001), demonstrated that expression of ERK1 dominant negative protein in the human osteoblast cell line HOB inhibited ERK / MAPK activity, resulting in decreased proliferation and differentiation but interestingly, inhibited adhesion, spreading and migration. The later was partly explained by the finding that β_1, $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrin expression

was also inhibited by the expression of the dominant negative ERK1 protein. These findings suggest that integrin expression and hence cell attachment, spreading and migration, are modulated by signal transduction elements that are demonstrated downstream effectors of integrin / ECM complexes and / or growth factor / growth factor receptor complexes (Lai *et al.* 2001). It is through these intracellular signalling pathways that processes including cellular proliferation, differentiation and migration have been linked with distinct integrins and their interactions with specific ligands (Leavesley *et al.* 1992; Leavesley *et al.* 1993; Miyamoto *et al.* 1996; Cowles *et al.* 2000; Gronthos *et al.* 2001; Lai *et al.* 2001; Maile *et al.* 2001).

1.3.1 Integrins present in bone

FCS contains multiple, diverse adhesive proteins such as fibronectin and VN. These ECM proteins adhere to culture substratum and mediate cellular attachment through interactions with integrins expressed on the cell surface. Osteoblasts express a diverse range of integrins including $\alpha_5\beta_1$, $\alpha_2\beta_1$, $\alpha_v\beta_1$ and $\alpha_v\beta_3$, although particular patterns of integrin expression is thought to be dependent on the particular stage of osteoblast differentiation (Bennett *et al.* 2001a; Bennett *et al.* 2001b). Gronthos *et al.*, (2001) recently suggested that the β_1-integrin subfamily may be involved in regulating the differentiation of bone marrow stromal precursor cells into mature, functional osteoblasts (Gronthos *et al.* 2001). While the α_v subunit is expressed in both osteoblasts and osteoclasts, the β_1 subunit appears to have the major functional role in osteoblasts whereas the β_3 subunit is predominant in osteoclasts. Indeed, the highest level of *in vivo* expression of $\alpha_v\beta_3$ integrins is in osteoclasts (Horton 1997; Duong *et al.* 2000; Bennett *et al.* 2001a). Schneller *et al.*, (1997) showed that mitogenicity was increased and activated PDGF receptor co-immunoprecipitated with $\alpha_v\beta_3$ integrin complexes when 3T3 fibroblasts were plated onto VN compared to collagen (Schneller *et al.* 1997). The $\alpha_v\beta_3$–VN relationship has been widely studied, especially in regards to its association with growth factor mediated cell function in various cell types.

In common with primary osteoblasts, SaOS-2 cell adhesion is mediated through the actions of integrin heterodimers involving α_v and β_1 subunits that bind with ECM proteins containing RGD sequences. These events are important for the attachment,

spreading, proliferation and differentiation of these cells on titanium and polystyrene surfaces (Degasne *et al.* 1999; Bennett *et al.* 2001b).

hMSCs have been shown to express α_1 (Bruder *et al.* 1998a) α_4, α_5 and β_1 (Gronthos *et al.* 2001) integrin subunits and of particular interest to this author, the $\alpha_v\beta_3$ and $\alpha_v\beta_5$ heterodimer complexes (Conget and Minguell 1999). The $\alpha_v\beta_3$ and $\alpha_v\beta_5$ integrins have been shown to bind VN via the RGD sequence thus mediating cellular attachment, reducing microvascular endothelial cells apoptosis (Isik *et al.* 1998) and, in the case of $\alpha_v\beta_3$, promoting IGF-I stimulated cell migration (Leavesley *et al.* 1992; Leavesley *et al.* 1993).

1.4 VITRONECTIN AND FIBRONECTIN

1.4.1 Vitronectin

Vitronectin (VN) is a 75 kDa multifunctional glycoprotein localised to the ECM and plasma. VN is involved in a wide variety of biological activities including promotion of cell adhesion, spreading, proliferation and migration. It is also involved in immune defence through interactions with complement complexes, and haemostasis through interactions with heparin. These functions are summarised in Figure 1.4 and have been reviewed by (Schvartz *et al.* 1999).

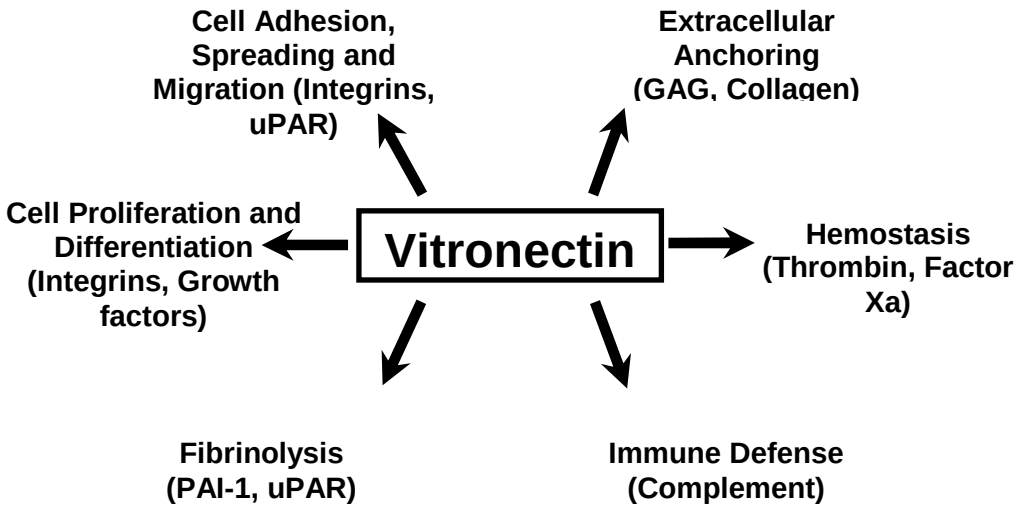


Figure 1.4. The major biological functions in which vitronectin is involved. Adapted from Schvartz *et al*., (1999).

The open reading frame (ORF) of VN, deduced from a human cDNA library, encodes for a 459 amino acid protein with a 19 amino acid signal peptide (Figure 1.5) (Schvartz *et al.* 1999).

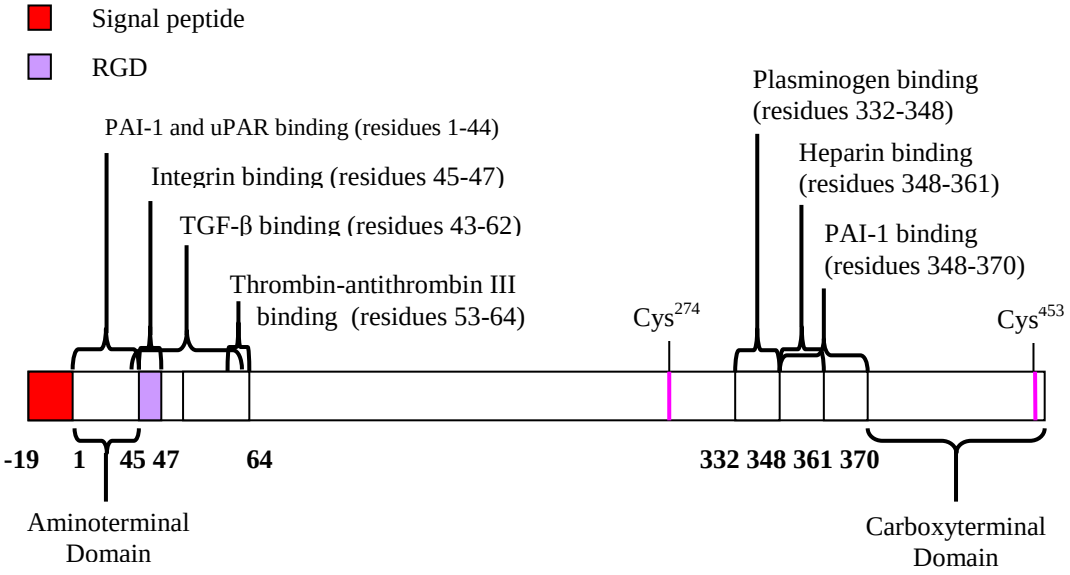


Figure 1.5. Schematic representation of the domain structure of vitronectin. The domain structure and localisation of the ligand binding domains on VN for; Plasminogen activator inhibitor-1 (PAI-1), urokinase plasminogen activator receptor (uPAR), integrins, TGF- β, thrombin-antithrombin III complex, plasminogen and heparin. Localisation of the cystine residues responsible for maintenance of multimeric quaternary structure via disulphide bond formation. Adapted from Schvartz *et al* (1999).

Plasma VN is largely synthesised in the liver and secreted into the circulation in a non-adhesive, monomeric 'native' conformation (Seiffert and Smith 1997). Native VN is structurally altered through interactions with a number of plasma proteins to form 'denatured' VN. Denatured VN can form multimers following endogenous cleavage of the 75 kDA native protein into 10 kDa and 65 kDa fragments, linked by a disulfide bond (Cys^{274}- Cys^{453}) (Figure1.5).

VN exists in the ECM in the multimeric form (Gibson and Peterson 2001). The amino terminal domain (residues 1-44), also known as the somatomedin B domain, has been shown to bind plasminogen activator inhibitor-1 (PAI-1) (Seiffert *et al.*

1994; Okumura *et al.* 2002) and urokinase plasminogen activator receptor (uPAR) (Wei *et al.* 1994; Okumura *et al.* 2002). PAI-1 competitively inhibits the VN / uPAR and VN / integrin interactions (Deng *et al.* 1996; Deng *et al.* 2001) and there is evidence to suggest it may also inhibit cell migration (Kanse *et al.* 1996). Directly adjacent to residues 1-44, is the Arg-Gly-Asp (RGD) integrin recognition sequence (aa 45-47) through which integrin dependent cellular attachment and spreading is mediated (Schvartz *et al.* 1999) (Figure 1.5). Cell attachment to VN occurs through integrin binding via the RGD motif and / or via VN-uPAR-uPA ± α_v integrin complex formation (Kjoller 2002). While PAI-1 competitively disrupts uPAR and integrin binding of VN, recent evidence suggests that PAI-1 can induce cell detachment from VN by interacting with active uPA bound to uPAR in a VN-independent process. In addition, this process is found to result in the inactivation and internalisation of uPAR-uPA-α_v integrin complexes (Czekay *et al.* 2003). These data demonstrate VN is able to facilitate cellular responses to specific ligands through co-localisation of the relevant receptors.

As discussed above, the RGD sequence facilitates the binding of a range of integrins to VN, including those expressed by bone cells such as $\alpha_v\beta_3$, $\alpha_v\beta_5$, $\alpha_5\beta_1$, $\alpha_2\beta_1$ (Bruder *et al.* 1998a; Conget and Minguell 1999; Gronthos *et al.* 2001). Of particular significance, TGF-β_1, a growth factor highly expressed in bone tissue, has been shown to bind to a region of VN between residues 43 and 62 (Figure 1.5). This is of particular importance because this interaction was also shown to inhibit both uPAR and integrin mediated cell adhesion (Schoppet *et al.* 2002). In addition, VN / TGF-β_1 complex formation did not interfere with TGF-β mediated signal transduction indicating a functional TGF-β / TGF-β receptor interaction (Schoppet *et al.* 2002). These data suggests a mechanism by which TGF-β initiated intracellular signalling could be mediated without interference from either uPAR or integrin intracellular signalling pathways. Interestingly, this interaction also provides a mechanism for the accumulation and storage of TGF-β in the ECM.

A region of acidic amino acids between residues 53 and 64 in VN is involved in binding the thrombin-antithrombin III complex and collagen (Figure 1.5). This region is also known as the polyanionic segment and is thought to associate with the

so-called polycationic segment (residues 348-376) via ionic interactions; this in turn stabilises the VN molecule as the folded monomer conformation found in plasma (Schvartz *et al.* 1999). The remainder of VN consists of six hemopexin repeats, which contain a plasminogen-binding site (residues 332-348), a heparin binding site (residues 348-361) and additional PAI-1 binding site (residues 348-370) (Kost *et al.* 1992) (Figure 1.5).

Francois et al, (1999) suggested that removal of multimeric VN complexes from the circulation was mediated by VN binding to endothelial cell surface glycosaminoglycans via VN's single functional heparin binding domain (residues 348-361) (Figure 1.5). Whether or not the bound multimeric VN is subsequently transported to underlying ECM or internalised and degraded remains unclear (Francois *et al.* 1999).

Another important family of proteins able to bind VN are the IGFs. Of particular interest to this author, VN has also been found to bind IGF-II but not IGF-1 (Upton *et al.* 1999). Moreover, it has been suggested that the binding interaction between IGF-II and VN may involve residues other than those involved in IGF-II binding to IGFBPs. This was indicated by the finding that des(1-6)-IGF-II, an IGF-II analogue with a much reduced affinity for IGFBPs, retained the ability to bind VN (Upton *et al.* 1999). This work, for the first time, demonstrated an interaction between VN and an important biologically active growth factor (Upton *et al.* 1999). Since then, as discussed above, TGF-β_1 has also been shown to bind VN (Schoppet *et al.* 2002). Inspired by the work by Upton and co-workers, Kricker *et al*., (2003) recently provided evidence that the IGFBPs could bind to VN and, in the case of IGFBP-5, enhance IGF-I stimulated biological effects, such as IGF-I mediated cell migration (Kricker *et al.* 2003). From the same laboratory Noble *et al*., (2003) demonstrated IGF-II pre-bound to VN enhanced IGF1R mediated MCF-7 breast cancer cell migration (Noble *et al.* 2003). The sites on VN of IGF-II and the IGFBPs binding remains to be elucidated, however, Kricker *et al*., (2003) demonstrated that the heparin binding domain of IGFBP-3 was critical for binding to VN (Kricker *et al.* 2003). Similarly, the heparin binding domain of IGFBP-5 is also critical for binding to VN (Nam *et al.* 2002). PAI-1 has also been shown to bind to VN and IGFBP-5, and VN is able to compete with PAI-1 for binding to IGFBP-5, suggesting a close

spatial relationship between the PAI-1 and IGFBP-5 binding sites on VN (Nam *et al.* 1997). Functionally, IGF-I / IGFBP-5 / VN complex formation has been shown to potentiate IGF-I mediated cell migration and DNA synthesis (Nam *et al.* 2002).

Interestingly, variation in the biological activity of VN bound to tissue culture plastic (TCP) has been shown to vary between TCP manufacturers which strongly suggests VN conformation is important (Underwood *et al.* 1993). The saturation limit for VN molecules attached to TCP substrates in a "side on" arrangement (as opposed to "end on) has been estimated at 210 ng/cm^2 (Pitt *et al.* 1987; Pitt *et al.* 1989). However, the amount of VN bound per cm^2 can be increased by exposing the TCP to higher concentrations of VN but this results in multilayering or reorientation of the molecules, ie. to "end on", demonstrated by interrogation of pre-bound VN with domain specific monoclonal antibodies. In the case of VN the conformation appeared not to change despite multilayering or reorientation (Underwood *et al.* 1993).

1.4.2 Fibronectin

Like VN, Fibronectin (FN) is a multifunctional glycoprotein present in plasma and tissue ECM. FN typically exists as a homo-dimer of two ~250 kDa disulfide linked subunits. However, the molecular weight (MW) of FN can vary depending on alternative splice variations (Tamkun *et al.* 1984). FN derived from plasma typically has a lower MW than cellular FN, is largely synthesised by hepatocytes in the liver and is present in plasma at ~300 µg/mL (Yamada and Kennedy 1979). A number of different integrins are capable of binding to FN and significant among these are the RGD binding integrins $\alpha 5\beta_1$ and $\alpha_v\beta_3$ (Ruoslahti 1988; Pankov and Yamada 2002). FN is also capable of binding a number of other ECM components including type I and type IV collagen, fibrin, heparin and heparan / condroitin / dermatan sulphate proteoglycans (Ruoslahti 1988; Romberger 1997). More recently FN has been shown to bind both IGFBP-3 and -5 (Gui and Murphy 2001). The interaction between IGFBP-5 and FN has been recently demonstrated to negatively modulate IGFBP-5's IGF-I dependent activity in mouse embryonic cell migration by enhancing proteolytic degradation of IGFBP-5. However, the identity of the particular protease or proteases mediating this phenomenon remains unresolved (Xu *et al.* 2004).

Additionally, FN has been shown to prevent apoptosis of mature osteoblasts and this effect was further enhanced by the addition of TGF-β_1. The authors found that FN and TGF-β_1 cooperated to regulate the survival of mature osteoblasts *in vitro* (Globus *et al.* 1998). Similar to VN, monolayers of FN molecules bound to tissue culture plastic have a saturation limit of 0.36 µg/cm^2 (Pitt *et al.* 1989). However, interrogation of high concentrations of pre-bound FN with monoclonal antibodies revealed that multilayering of the FN molecules was possibly occurring, although it remained unclear whether this result was an artefact caused by steric hindrance of antibody binding to FN molecules (Underwood *et al.* 1993).

1.5 INSULIN-LIKE GROWTH FACTORS

The IGFs, IGF-I and IGF-II, are growth-promoting polypeptides which have both anabolic and mitogenic effects on cells and have essential roles in growth and development. IGF-I and II are both single chain polypeptides, similar in amino acid sequence (62%) and structure and bear substantial homology (40%) with proinsulin (Yu and Berkel 1999). IGF-I and IGF-II are synthesised as pre-proteins and circulate as 70 and 67 amino acid mature peptides, respectively. IGF-I is 7.6 kDa basic peptide while IGF-II is slightly acidic and has a molecular weight of 7.5 kDa. The biological actions of the IGFs are mediated through 2 IGF receptors. These are the type 1 IGF receptor (IGF1R) and the type 2 IGF receptor (IGF2R). The type II receptor is also known as the cation-independent mannose-6 phosphate receptor (CIMPR). Both IGF-I and IGF-II bind to the IGF1R with similar high affinity, whereas only IGF-II binds with high affinity to the CIMPR (Kiess *et al.* 1994). Interestingly, as discussed above, IGF-II has been shown to directly interact with VN (Upton *et al.* 1999), as has TGF-β_1 (Schoppet *et al.* 2002). Both IGF-II and TGF-β_1 interact with the IGF2R / CIMPR (Dennis and Rifkin 1991; Kiess *et al.* 1994; Ghosh *et al.* 2003). Additionally, the CIMPR has been shown to mediate the activation of latent TGF-β_1 by forming complexes with plasminogen and uPAR (Godar *et al.* 1999) the latter of which is also known to interact with VN (Wei *et al.* 1994; Okumura *et al.* 2002). However, no studies have yet looked at possible trimeric, IGF-II / VN / TGF-β_1 complex formation, nor the possible functional consequences of such an interaction.

IGF action is also regulated by the 6 so-called IGF binding proteins (IGFBP 1-6). IGFBPs facilitate the availability and action of the IGFs by extending IGF half life, transporting the IGFs in serum, mediating IGF binding to their receptors and importantly, localising IGFs in tissues. Due to differences in structure, post-translational modifications, and expression patterns, each of the IGFBPs are associated with unique functions (Wood 1995).

1.5.1 Insulin like growth factors and hMSCs

The expression of IGF-I mRNA in hMSCs has been shown to increase in response to stimulation by TGF-β_1 (Kveiborg *et al.* 2001a). However, no changes in IGF-II mRNA could be detected following identical treatment. Regulation of IGF-I synthesis by TGF-β_1 on the other hand has been demonstrated at the transcriptional level (Kveiborg *et al.* 2001a). These findings support the hypothesis that TGF-β_1 has a stimulatory effect on hMSC cell proliferation by inducing a localised increase in IGF-I levels, which act in a paracrine fashion. However, mature human osteoblasts obtained from trabecular bone explants were found to consistently express both IGF-I and -II mRNA transcripts, while transformed osteoblast cell lines were found to express neither IGF-I nor IGF-II mRNA transcripts (Okazaki *et al.* 1995).

Glucocoticoids, such as dexamethasone (Dex), are well known to stimulate rodent and human osteoprogenitor cell differentiation (Cheng *et al.* 1994; Haynesworth *et al.* 1996; Bruder *et al.* 1997b; Jaiswal *et al.* 1997). Jia and Heersche (2002) have investigated which components of the IGF system, ie IGF-I and II, IGFBPs and IGFRs, are involved in Dex-induced *in vitro* rat osteoprogenitor differentiation. Significantly, they found that IGF-I transcripts were down-regulated in cultures treated with Dex, suggesting that IGF-I down-regulation is a consequence of the differentiation process, or that differentiation is triggered by a reduction of local IGF-I concentration and therefore reduced IGF-mediated cell proliferation (Jia and Heersche 2002). This supports the earlier findings of (Delany and Canalis 1995) and similar findings in human marrow stromal cells (Cheng *et al.* 1998), but seemingly contradicts an earlier report from the same authors that IGF-I and -II stimulated osteoprogenitor proliferation and differentiation (Jia and Heersche 2000). However, the later study also revealed that IGFBP-4, which is known to be inhibitory to IGF-I induced osteoblastic cell proliferation (Grellier *et al.* 1996; Gustafsson *et al.* 1999),

was not highly transcribed in immature, actively growing osteoprogenitor colonies. In contrast, IGFBP-4 was highly transcribed in older, differentiating cultures in which osteoid was accumulating. Furthermore, transcription of IGFBP-3, which is known to have stimulatory effects on IGF mediated cell proliferation (Conover and Kiefer 1993; Ramagnolo *et al.* 1994), was upregulated in immature, proliferating cultures but was found to be down-regulated as cultures differentiated and aged. Taken together these findings suggest that IGFBP-3 may mediate storage of IGF-I in immature cultures and as synthesis of IGF-I and IGFBP-3 decreases, synthesis of IGFBP-4 coincidentally increases, thus competing for remaining IGF-I and reducing its availability and mitogenic activity. Recently, Walsh *et al*., (2003) found that exogenous IGF-I did not support hMSC proliferation, nor did it induce osteoblast marker expression. However, in the assay systems used in this study the authors added the IGF-I to the hMSCs in solution and in the presence of 15% FCS. Thus, any effect of exogeneous IGF-I may have been masked by responses to the many growth factors and mitogens in the FCS (Walsh *et al.* 2003).

1.5.2 Insulin like growth factors: Localisation and role in bone remodelling

IGF-I and -II are among the most abundant growth factors synthesised by osteoblasts and are largely present in bone matrix as part of a larger protein complex with the IGF binding proteins (Bautista *et al.* 1990; Bautista *et al.* 1991; Mohan and Baylink 1991). Localisation of the IGFs in bone tissue is primarily due to the ECM binding capabilities of the IGFBPs, particularly IGFBP-5. IGFBP-5 has been shown to accumulate in bone ECM and is capable of binding hydroxyapatite, the mineral component of bone (Bautista *et al.* 1991). Many studies, however, have investigated the effect of various cytokines and hormones on the expression of the IGFs by osteoblasts. For example triiodothyronine (T3) has been shown to have little effect on IGF-I expression, but stimulated a doubling of the number IGF1Rs available for the binding of IGF-I on the surface of cultured human osteoblasts (Pepene *et al.* 2001). Similarly, the stimulatory effect of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) on the proliferation of murine MC3T3-E1 cells was found to be due to increased expression of the IGF1R at the cell surface in the absence of any increase in IGF-I (Hakeda *et al.* 1991). Interestingly, Dex induced osteoblast differentiation of human bone marrow stromal cells resulted in a decrease of IGF-I, IGFBP-3, IGFBP-5 and IGFBP-6 synthesis and secretion, and an increase in IGF-II and IGFBP-2 levels

(Cheng *et al.* 1998). This is consistent with the observed reduction in proliferation of osteoblast precursors exposed to Dex, since IGF-I, IGFBP-3 and IGFBP-5 are known to have mitogenic effects on cells (Jones *et al.* 1993; Ramagnolo *et al.* 1994). However, the increase in IGF-II, along with the decrease in IGFBP-6 levels observed in response to Dex treatment, is interesting because both IGF-I and IGF-II have been associated with enhancement of osteoblast differentiation processes (McCarthy *et al.* 1989c; a; Jia and Heersche 2000). IGFBP-6 has been shown recently to have a much higher affinity for IGF-II than for IGF-I (Kricker *et al.* 2003) and therefore down-regulation of IGFBP-6 would also increase the availability of IGF-II in the local microenvironment. However, few studies have investigated the spatial expression of the IGFs in relation to cells of the BMU.

1.6 INSULIN LIKE GROWTH FACTOR BINDING PROTEIN-5

IGFBP-5 was first isolated from human bone tissue in 1991 by Bautista and co-workers (Bautista *et al.* 1991). Cleavage of a 20 amino acid signal peptide yields a 29 kDa mature IGFBP-5 peptide of 252 amino acids, which is secreted from the cell (Figure 1.6). IGFBP-5, like the other IGFBPs, has three distinct domains, each of similar size: an N-terminal domain, which contains the primary binding site for IGFs I and II; a mid-region, in which each IGFBP has an almost unique sequence and where most post-translational modifications of the protein occur; and a C-terminal domain which contains regions responsible for binding the ECM, the 85 kDA leucine rich glycoprotein known as the acid labile subunit (ALS) (Baxter *et al.* 1989) and cell membrane proteins (Figure 1.6). It is thought that the unique sequence within the mid-region confers each IGFBP with unique functions, although this still requires definitive proof. Most of the proteolytic sites identified thus far are located in this region, as are 4 O-glcosylation sites ($Thr^{103,}$ $Thr^{104,}$ Thr^{111} and Thr^{152}) (Conover and Kiefer 1993) and 12 phosphorylation sites (Coverley and Baxter 1997) (Figure 1.6). IGFBP-5 susceptibility to proteolytic degradation is potentially altered due to the proximity of many of these post-translational modifications to known proteolytic sites (Bach 1999) (Figure 1.6). IGFBP-5 binds to ECM proteins such as thrombospondin, osteopontin (Nam *et al.* 2000) and VN (Nam *et al.* 2002) with high affinity. It is thought that IGFBP-5 binding to the ECM is mediated by ionic interactions (Jones *et al.* 1993) with 2 regions that contain several basic amino acid

clusters; Arg^{201}-Arg^{218} and Ala^{131}-Thr^{141}. Of these regions, residues 201-218 exhibit ~ 4-fold more potent ECM binding than residues 131-141 (Parker *et al.* 1996). Recently, residues 77-81 in the amino terminus and residues 217 and 223 in the carboxy terminus of IGFBP-3 were identified as critical residues involved in high affinity binding of IGF-I (Yan *et al.* 2004). Similarly, residues 203 and 209 in the carboxy terminus of IGFBP-5 have been confirmed to be critical for the IGFBP-5 / IGF-I interaction (Allan *et al.* 2006). Amino acids 201-218 have also been shown to regulate IGFBP-5 binding to hydroxyapatite (the mineral component of bone) (Campbell and Andress 1997) and the ALS (Figure 1.6).

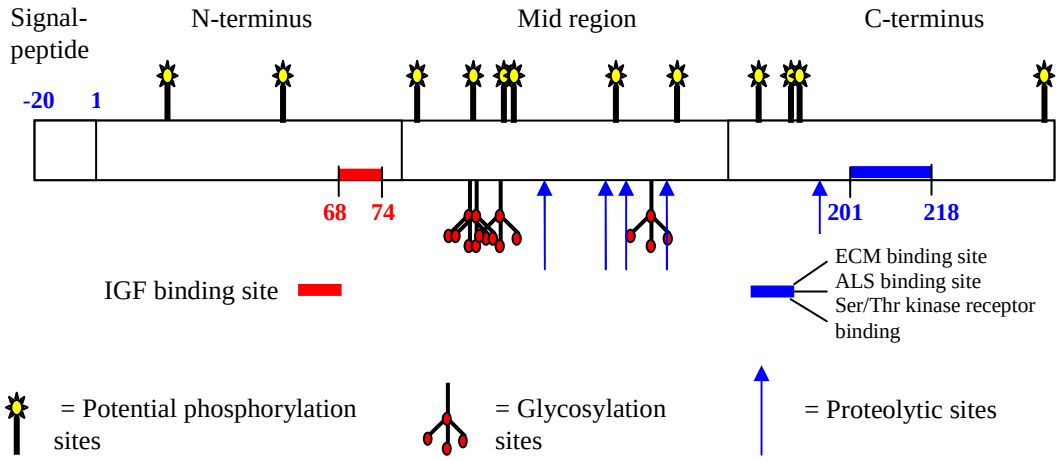


Figure 1.6. Schematic representation of the insulin like growth factor binding protein 5 (IGFBP-5) domain structure. Adapted from (Schneider *et al.* 2002)

The ALS is known to participate in the formation of a high molecular weight (150 kDa) ternary complex with either of the IGFs and IGFBP-3, and is thought to i) protect IGFBP / IGF complexes from proteolytic degradation and to ii) regulate passage of IGFBP-3 / IGF-I complexes from the circulation into target tissues. More recently IGFBP-5 has also been shown to be capable of participating in ternary complex (130 kDa) formation with the IGFs and the ALS (critical residues Lys^{211}, Arg^{214}, Lys^{217} and Arg^{218}) (Twigg and Baxter 1998; Twigg *et al.* 1998; Firth *et al.* 2001). This can be explained by close sequence similarity between the carboxyl domains of IGFBP-3 and IGFBP-5. The ALS is primarily produced by the liver and is found mainly in serum. Significantly, thus far just 1 study has looked for

expression and production of ALS by bone cells (Kanzaki *et al.* 1995). The authors found that ALS was not released into the conditioned media by either normal human osteoblasts, or the osteoblast-like, SaOS-2 osteosarcoma cell line. Curiously, studies examining the presence of the ALS have yet to be performed on natural bone, or osteoblast-derived ECM, major reservoirs of both IGFBP-5 and IGFs (Bautista *et al.* 1990; Bautista *et al.* 1991; Hakeda *et al.* 1996; Campbell and Andress 1997; Govoni *et al.* 2005). Since the ALS in the circulation is involved in regulating the availability of IGFBP / IGF complex to the tissues, a similar mechanism could exist in bone. For example the ALS might protect bone matrix bound IGFBP-5 / IGF complex from proteolytic degradation until osteoclast resorption creates an acidic environment that can degrade the ALS and in turn allow specific proteases access to the IGFBP-5 / IGF complex and release IGF into the local microenvironment.

IGFBP-5 also has been shown to interact with the protease inhibitor, plasminogen activator inhibitor-1 (PAI-1) (Nam *et al.* 1997). Jones *et al*., (1993) found that the growth stimulatory effect of IGF-I on fibroblasts was potentiated when IGFBP-5 was present in cell culture substrata. However, no effect on IGF-I stimulated growth was observed when IGFBP-5 alone was present in the medium. Rather, it was degraded to a 22 kDa fragment, thus suggesting that IGFBP-5 bound to ECM is protected from proteolytic degradation and is able to localise IGF-I to the ECM where it may mediate cellular growth responses (Jones *et al.* 1993).

1.6.1 Insulin like growth factor binding protein-5 and hMSCs

While many studies have examined the interactions and effects of IGFBP-5 in osteoblasts, few studies have addressed the interactions or effects of IGFBP-5 in hMSCs. Nonetheless, it has been shown that hMSCs do express both IGFBP-5 mRNA and protein and that this expression can be negatively regulated by treatment of hMSC cultures with dexamethasone. Indeed, IGF-I expression is down-regulated in hMSCs treated with dexamethasone (Cheng *et al.* 1998). Thus, considering that together IGFBP-5 and IGF-I are known to have mitogenic effects on osteoblast cell proliferation (Cheng *et al.* 1998) a possible mechanism for the negative effect on hMSC proliferation by dexamethasone is suggested. In addition, IGFBP-5 has recently been shown to enhance IGF-I mediated hMSC chemotactic migration (Fiedler *et al.* 2006).

1.6.2 Insulin like growth factor binding protein-5: Localisation in bone

IGFBP-5 was originally isolated from human bone by Bautista et al (1991) using a HA affinity matrix, leading to the hypothesis that IGFBP-5 association with HA was a potential mechanism of localisation in mineralised tissue. However, IGFBP-5 has also been shown to bind type III and type IV collagens, laminin (Jones *et al.* 1993), fibronectin and VN (Nam *et al.* 2002). *In vitro* studies in human fibroblast monolayer cultures have shown that glycosaminoglycans such as heparin, heparan sulphate and dermatan sulphate can protect IGFBP-5 from proteolytic degradation (Arai *et al.* 1994). These data suggest the potential mechanism of regulation of IGFBP-5 function and / or accumulation of IGFBP-5 in bone tissue may be complex and manifold.

PTH and prostaglandin E_2 occupy key roles in bone remodelling and hence have also been examined for specific effects on IGFBP-5 degradation. These hormones induce the breakdown of IGFBP-5 from the bone ECM of cultured mouse calvariae, resulting in the accumulation of 21 kDa immunoreactive fragments in conditioned media (Hakeda *et al.* 1996). As IGF-I and -II have also been shown to accumulate in bone matrix, the demonstrated presence of IGFBP-5 suggests a possible mechanism for localising the IGFs in bone tissue until required by cells.

1.6.3 Insulin like growth factor binding protein-5: Role in bone remodelling

The presence of IGFBP-5 in bone matrix suggests that IGFBP-5 has a role in normal bone physiology through direct, or indirect, stimulation of osteoblast behaviour. As discussed above, IGFBP-5 associates with the bone mineral (HA) matrix which may function to sequester and localise IGF-I and -II in bone tissue for potential release in bone remodelling (Bautista *et al.* 1991). Of the 6 IGFBPs, IGFBP-5 binds the IGFs with the highest affinity. Interestingly, IGFBP-5 binds IGFs with a 50 fold greater affinity than the IGF1R (Cohick and Clemmons 1993). Recently, our laboratory demonstrated that IGF-I mediated cellular migration is significantly enhanced through the formation of a trimeric complex of IGF-I, IGFBP-5 and VN (Kricker *et al.* 2003). These data suggest IGF-I mediated cell activity may be regulated in bone through an additional mechanism since VN, a ubiquitous ECM protein of all connective tissue, is co-localised with IGFBP-5 and IGF-I present in high concentration in bone tissue (Bautista *et al.* 1991); (Andress and Birnbaum 1992;

Hakeda *et al.* 1996; Franchimont *et al.* 1997a; Franchimont *et al.* 1997b). However, whether this trimeric complex naturally occurs in bone tissue remains to be verified.

In addition to facilitating IGF mediated cell-signalling pathways, IGFBP-5 mediates IGF independent cell responses. A 23 kDa fragment and a carboxytruncated form of IGFBP-5 have been shown to have mitogenic effects independent of IGF-I in neonatal mouse (Andress and Birnbaum 1992) and human (Mohan *et al.* 1995b) osteoblast-like cells. In addition, IGFBP-5 stimulated proliferation of osteoblasts derived from IGF-I knockout mice, even in the presence of IGFBP-4, a potent inhibitor of IGF actions in osteoblasts, further demonstrating that IGFBP-5 has independent biological activity (Miyakoshi *et al.* 2001). Andress (1995) found that IGFBP-5 binds and is internalised with a 420 kDA membrane protein in mouse osteoblasts. This "ligand-induced internalisation" appears to require the basic region at 201-218 in IGFBP-5 (Andress 1995). A subsequent study revealed that intact IGFBP-5 and the IGFBP-5 fragments 1-169 and 201-218 stimulated phosphorylation of serine residues present in this 420 kDA membrane protein and this putative 'BP-5 receptor' has demonstrated protein kinase capability; phosphorylating casein *in vitro* (Andress 1998). This indicates that at least in osteoblasts, IGFBP-5 acts in an IGF independent manner, possibly with its own signal transduction pathway (Andress 1998). However, the physiological role of this interaction remains to be elucidated. While the identity of this putative IGFBP-5 receptor is unknown, the authors discussed the existence of only one other 400 kDa membrane protein with serine / threonine kinase activity, the type V TGF-β receptor (O'Grady *et al.* 1992). However, treatment with TGF-β was not able to stimulate IGFBP-5 receptor phosphorylation, indicating that while the putative IGFBP-5 receptor and the type V TGF-β receptor are functionally similar (phosphorylate casein on serine residues), they have distinct ligand binding characteristics (Andress 1998). In addition, co-immunoprecipitation and immunofluorescence studies further demonstrate that i) IGFBP-5 interacts with a nuclear transcription modulator protein known as 'Four and a Half LIM Protein 2' (FHL2) and that ii) both FHL2 and IGFBP-5 localise to the nucleus in human bone cells (Amaar *et al.* 2002). While the functional significance of the above observations is unclear, experimental data reported by others clearly indicate that IGFBP-5 acts as a growth factor that translocates to the nucleus via a signal-dependent β importin-mediated pathway (Schedlich *et al.* 1998;

Schedlich *et al.* 2000). Moreover, the androgen receptor is co-activated by FHL2 (Muller *et al.* 2000), This further suggests that the FHL2 / IGFBP-5 interaction may play a role in the modulation of osteoblast proliferation or differentiation processes (Figure 1.7). In fact recently, FHL-2, IGFBP-5 and a disintegrin and metalloprotease-9 (ADAM-9) (a known IGFBP-5 protease) were found to be co-ordinately regulated during osteoblast differentiation in response to either dexamethasone, TGF-β_1 or bone morphogenetic protein-7 (BMP-7) (Govoni *et al.* 2006).

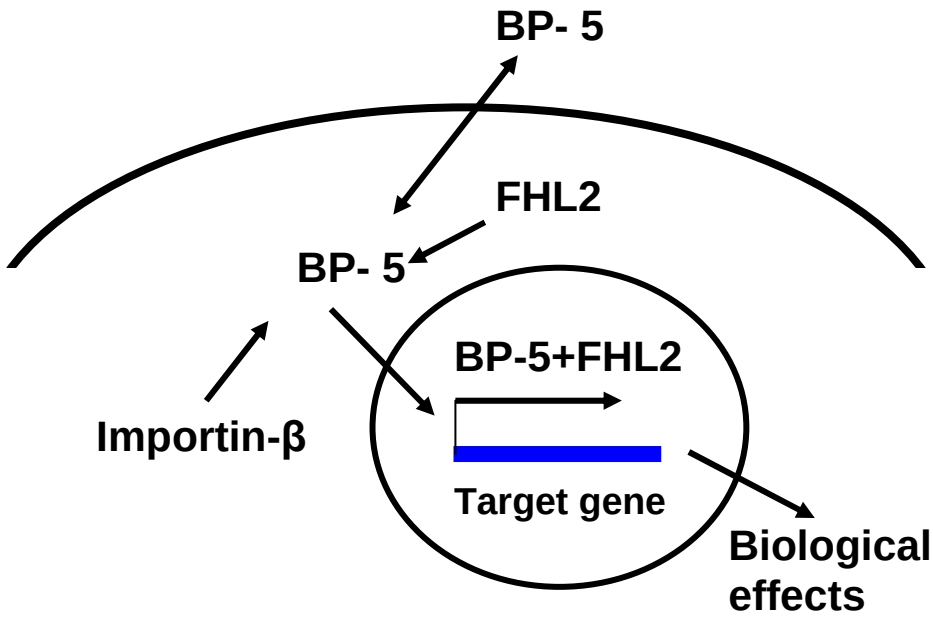


Figure 1.7. Model of IGF independent action of IGFBP-5 in osteoblasts. In the proposed IGF-independent mechanism, IGFBP-5 from the cytoplasm, or an extracellular source, enters the nucleus via its nuclear localisation sequence and the nuclear transporter, importin-β. IGFBP-5 may bind to FHL2, a transcription modulator, in the cytoplasm and shuttle FHL2 into the nucleus or bind FHL2 in the nucleus to stimulate transcription of target genes. Adapted from (Mohan and Baylink 2002).

1.7 TRANSFORMING GROWTH FACTOR-β_1

TGF-β_1 is the prototypical member of the TGF-β superfamily, which currently consists of over 30 members. These have been further characterised into the inhibin / activin family, the mullerian inhibitory substance (MIS) family, the bone morphogenic protein family and the TGF-β family (Bonewald 1999). All TGF-β

family members are potent stimuli of cell functions including proliferation and differentiation. In mammals there are 3 isoforms of TGF-β, TGF-β_1, TGF-β_2 and TGF-β_3, which exist predominantly as homodimers. However, heterodimers of TGF-$\beta_{2/3}$ and TGF-$\beta_{1/3}$ have also been isolated (Bonewald 1999). Dramatic differences are evident in the tissue distribution of the various TGF-β isoforms. For example, the TGF-β_2 isoform represents about 50% of total TGF-β present in the kidney (Marra *et al.* 1996), while in the prostate there is a 20-30 fold over-expression of the TGF-β_2 isoform relative to the TGF-β_1 isoform. In contrast, 80 to 90% of TGF-β in bone is the TGF-β_1 isoform (Bonewald 1999).

The TGF-β family members mediate diverse biological effects through binding with the Type I, II or III TGF-β receptors. Each isoform of TGF-β binds with different affinities to each of the TGF-β receptors suggesting diverse combinations of TGF-β isoforms and TGF-β receptor provide potential for varied biological effects (Bonewald 1999). For example, the Type-I TGF-β receptor binds TGF-β_2 with much higher affinity than either TGF-β_1 or TGF-β_3 (Cheifetz *et al.* 1986), while the Type II TGF-β receptor bind TGF-β_1 and TGF-β_3 with a 100 fold higher affinity than TGF-β_2 (Lin *et al.* 1992). Most cells in the body express receptors for TGF-β, suggesting responses to TGF-β must be tightly regulated. Further, most cells secrete TGF-β in its latent, or inactive form (Hyytiainen *et al.* 2004). However, the predominant reservoirs of TGF-β in the body are platelets, bone and serum (Seyedin *et al.* 1985; O'Connor-McCourt and Wakefield 1987; Wakefield *et al.* 1988), although TGF-β within these reservoirs exists in the latent or inactive form.

Like the IGFs, TGF-β_1 exists in bone primarily as part of a functionally inactive larger protein complex. This complex consists of a 25 kDa TGF-β homodimer, which following proteolytic cleavage remains non-covalently associated with a 75 kDa propeptide homodimer, referred to as the latency-associated peptide (LAP). Three known isoforms of LAP correspond to each mature TGF-β isoform to which it is complexed ie. LAP-β_1, LAP-β_2 or LAP-β_3. This complex, termed the small latent complex, represents approximately 50% of the latent TGF-β produced by osteoblasts. However, the small latent complex is also produced by other cell types and as such may serve a unique function as a free circulating, more activatable form (Bonewald *et al.* 1991; Bonewald 1999). Interestingly, LAP-β_1 and LAP-β_3 have

been shown to contain an RGD sequence and potentially bind to all of the α_v integrins, including $\alpha_v\beta_1$, $\alpha_v\beta_5$ (Munger *et al.* 1998), $\alpha_v\beta_3$ (Ludbrook *et al.* 2003), $\alpha_v\beta_6$ (Munger *et al.* 1999) and $\alpha_v\beta_8$ (Mu *et al.* 2002). In LAP-β_2 the arginine is substituted with a serine residue (SGD) and is not a ligand for any of these integrins (Ludbrook *et al.* 2003). While the function of these interactions is not currently understood, it is possibile that i) α_v integrins localise TGF-β at the cell surface to be activated by specific proteases such as MMP-1 (Mu *et al.* 2002) or MMP-2 (Brooks *et al.* 1996) or ii) LAP / integrin ligation initiates α_v integrin specific intracellular signalling events. The propeptide, LAP, disulfide linked to the 190 kDa "latent transforming growth factor-β-binding protein 1" (LTBP1) (Kanzaki *et al.* 1990; Dallas *et al.* 1995), is a member of a large family of ECM proteins which include fibrillins 1 and 2 and LTBPs 1-4 (Sinha *et al.* 1998).

1.7.1 Transforming growth factor β_1 and hMSC's

TGF-β_1 is a potent mitogen of hMSCs *in vitro* through the induction of IGF-I (Kveiborg *et al.* 2001b) and has also been found to stimulate the proliferation of mature osteoblasts. However, in the presence of calcitrol, TGF-β_1 stimulates the production of bone matrix associated proteins such as alkaline phosphatase and type 1 collagen and inhibits the expression of osteocalcin, required for matrix mineralisation and terminal differentiation of mature osteoblasts (Kassem *et al.* 2000). Similarly, TGF-β_1 has also been shown to inhibit bone morphogenetic protein 2 (BMP-2) mediated osteoblast precursor differentiation (Spinella-Jaegle *et al.* 2001). Thus the effects of TGF-β_1 on hMSCs are complex and unclear.

Intriguingly, activated TGF-β_1 is also capable of binding to VN at a site in close proximity to VN's RGD motif, inhibiting both uPAR and α_v-integrin dependent cell attachment (Schoppet *et al.* 2002). Thus TGF-β_1 may also effect uPAR and integrin mediated signal transduction pathways. This finding is of particular interest because it suggests TGF-β_1 may be immobilised on various substrates as part of an ECM / growth factor complex. Interestingly, this and the findings of Kricker and co-workers that IGF-I could bind VN via select IGFBPs (Kricker *et al.* 2003), highlights the prospect of incorporating both TGF-β_1 and IGF-I into pre-bound substrate coatings for bone tissue engineered therapeutics. While it is widely held that TGF-β_1 induces

differentiation of osteoblasts, in 2001 Kveiborg and co-workers reported that TGF-β_1 induced hMSCs to express IGFBP-3 and IGF-I thus inducing hMSC proliferation (Kveiborg *et al.* 2001a). TGF-β_1 and IGF-I were combined in a recent study of periosteal hMSCs in an *in vitro* organ culture model of chondrogenisis, and it was found that the combination of TGF-β_1 and IGF-I stimulated an enhanced proliferative response in early chondrogenesis and over 6 weeks resulted in greater cartilage growth compared to the responses to either of the individual growth factors alone (Fukumoto *et al.* 2003). While the above was an agarose suspension periosteal organ culture system designed to induce chondrogenesis, no other investigations have examined the response of bone marrow derived hMSCs with combined TGF-β_1 and IGF-I in a pre-bound format with VN.

1.7.2 Transforming growth factor β_1: Localisation in bone

Dallas and co-workers (2002) recently demonstrated that osteoclast-mediated proteolysis of the LTBP1 complex released latent TGF-β_1 from cultured primary osteoblast-derived ECM, *in vitro*. In addition, purified forms of the serine proteases, elastase and plasmin, and the matrix metalloproteinases (MMP) MMP2 and MMP9 were shown to cleave LTBP1, releasing latent TGF-β_1 from the ECM (Dallas *et al.* 2002). Earlier studies demonstrated that active TGF-β_1 is dissociated from the LTBP-1 complex at low pH suggesting that the acidic microenvironment produced within the resorption zone of active osteoclasts may be another mechanism through which active TGF-β_1 can be liberated (Pfeilschifter and Mundy 1987; Pfeilschifter *et al.* 1990; Hosoi *et al.* 1996). Indeed, activated TGF-β_1 has been immuno-localised to osteoclast resorption lacunae and adjacent ECM in mice calvariae. TGF-β_1 has been shown, *in vitro*, to induce mature osteoblasts to express both itself and the LTBP1, which are then assembled inside the cell and secreted into newly formed bone matrix (Miyazono *et al.* 1991), presumably to await release and activation at a later point by resorbing osteoclasts. However, to the best of this author's knowledge the distribution or spatial relationship of the small latent complex and the large latent binding protein complex is entirely uncharacterised. Furthermore the fate, or function, of osteoclast activated TGF-β_1 *in vivo* is also unknown. Does osteoclast activated TGF-β_1 bind cell surface receptors on osteoclasts, osteoblasts, or the precursors of these cell types? Which receptors do they interact with? *In vivo* co-localisation and *in vitro* cell signalling studies may help to answer these questions

and assist the elucidation of the role of TGF-β_1 in the sequence of events between bone resorption by osteoclasts and subsequent bone formation by osteoblasts.

1.7.3 Transforming growth factor β_1: Role in bone remodelling

The largest source of TGF-β_1 in the body is bone tissue, although it exists largely as an inactive latent complex. Release of activated TGF-β_1 occurs in actively resorbing bone and this activation has been shown to occur by a number of mechanisms, (discussed above). Local release of TGF-β_1 from bone matrix by osteoclasts during bone resorption may be an important regulatory mechanism of bone cell activity (Bonewald and Mundy 1990). TGF-β_1 has been shown to inhibit osteoclastogenesis through the stimulation of marrow stromal and osteoblastic cells to produce osteoprotegerin (OPG). This in turn binds to receptor activator of NF-κβ ligand RANK-L, preventing activation of RANK on the surface of osteoclastic cells (Lacey *et al.* 1998). However, others have demonstrated that osteoclast differentiation processes and RANK expression in the murine monocytic cell line RAW 264.7 is increased when exposed to TGF-β_1 (Yan *et al.* 2001). Thus the net effect of exposure to TGF-β_1 on bone resorption may be regulated by balanced responses between marrow stromal / osteoblastic cells on the one hand, and pre-osteoclasts / osteoclasts on the other.

Karsdal *et al*., (2001) reported that osteoblasts cultured on the surface of bone slices, mimicking the osteoblastic 'bone lining cells' found *in vivo*, underwent a morphological change from cuboidal, or cobblestone appearance, to a highly elongated shape when exposed to TGF-β_1. This shape change exposed the underlying bone surface, allowing co-cultured osteoclasts access to the substratum, which in turn facilitated resorption pit formation. In addition, the authors found that TGF-β_1 induced osteoblast elongation was dependent on p38 MAP kinase activity and MMP 13 (collagenase 3) expression. Although up regulation of MMP 13 expression by TGF-β_1 induced signal transduction pathways was independent of the p38 MAP kinase pathway, TGF-β_1 regulation of osteoblast morphology is affected by multiple intra-cellular signalling pathways (Karsdal *et al.* 2001). As discussed above, the latent TGF-β complex may be released from the ECM and subsequently activated by either specific proteolytic cleavage, or exposure to extremes in pH. Activated TGF-β itself can regulate the activation of the latent complex by

stimulating protease inhibitor production, such as PAI-1 and tissue inhibitor metalloproteases (metalloprotease inhibitors) (Laiho *et al.* 1986; Bonewald and Mundy 1990) and inhibition of plasminogen activator (Laiho *et al.* 1987), urokinase and collagenase (Bonewald and Mundy 1990).

TGF-β_1 also stimulates ascorbate transport in osteoblasts by increasing the rate of synthesis of either Na^+ ascorbate co-transporters or proteins that interact with and regulate existing transporters in the cell membrane to increase ascorbate uptake (Dixon and Wilson 1992). Because ascorbate (Vitamin C) is well known as an essential requirement for osteoblastic differentiation and collagen synthesis (Murad *et al.* 1981; Jaiswal *et al.* 1997; Coelho and Fernandes 2000) this may be a mechanism through which TGF-β_1 enhances matrix synthesis by osteoblastic cells.

1.8 MATRIX METALLOPROTEINASES

A potential problem facing the successful culture of primary bone cells on pre-bound protein complexes is the possible degradation of the matrix protein / growth factor substrate by cellular expression of various proteases. It is well known that actively migrating cells produce a range of proteases in order to facilitate migration through the ECM; this is particularly evident in pathogenic processes such as facilitation of metastatic tumour dissemination (Sternlicht and Werb 2001) and in normal processes such as bone remodelling. In the normal, healthy *in vivo* context protease expression is highly regulated, whereas, in the *in vitro*, context especially in serum-free conditions, regulatory mechanisms may be absent and the effect of protease expression on pre-bound protein complexes, such as that of VN an IGFBP and IGF-I, may result in destruction of the protein substrate causing less efficient growth rates of cells. On the other hand proteolytic degradation of some of the components, such as IGFBPs, may be required for mediation of cellular responses by liberation of latent growth factors.

Among the most prevalent proteases expressed by osteoblasts are the Matrix Metalloproteinases (MMPs), in particular MMP-2 and MMP-9. These 2 proteases have been demonstrated as being able to liberate active TGF-β_1 from ECM by proteolytic cleavage of the latent transforming growth factor-β-binding protein 1

(LTBP1) complex (Dallas *et al.* 2002). MMP-3 (stromelysin 1), also expressed by osteoblasts (Bord *et al.* 1998; Breckon *et al.* 1999), was recently found to play a role in the regulation of cellular proliferation by disruption of IGF-I / IGFBP-3 complexes, thus liberating active IGF-I leading to activation of the IGF-I receptor (Fowlkes *et al.* 2004). Similarly, some members of the ADAMs (a disintegrin and metalloprotease) family of proteins, a subfamily of the MMPs, are also able to cleave IGFBP-3 and / or IGFBP-5 (ADAM 12-S and ADAM 9 respectively) (Loechel *et al.* 2000; Mohan *et al.* 2002). More recently, MMP-7 has been shown to be capable of proteolytically degrading all of the IGFBPs (Nakamura *et al.* 2005). However, Payet and co workers demonstrated in 2003 that amino and carboxy terminal fragments of IGFBP-3 bind to IGF-I and -II with high affinity and inhibit IGF mediated phosphorylation of the IGFIR and IGF-II interaction with the CIMPR (Payet *et al.* 2003). Thus the biological function of proteolytic degradation of the IGFBPs in relation to IGF bioavailability remains unclear.

Plasmin is a well known VN protease which has been shown to activate MMP-2 in conjunction with MT1-MMP (Monea *et al.* 2002). In addition, MT1-MMP is known to degrade VN and clips pro α_v integrins to give the active form. The authors found that inhibition of MT1-MMP with AG3340 (Prinomastat) protected VN from degradation by MT1-MMP over the short term, therefore increasing cell motility. However over the long term (~24 hrs) inhibition of MT1-MMP resulted in the cessation of cell migration, as cell surface α_v integrins were replaced with inactive pro α_v integrins. MT1-MMP half life is short (only a few hrs), whereas α_v integrins have a relatively long half life at the cell membrane (~24 hrs) on MCF-7 cells (Deryugina *et al.* 2003). A number of questions arise from the above information: do cells cultured on pre-bound matrix / growth factor complexes express proteases which degrade the protein substrate and thus regulate cell function? If so which ones? And if these proteases are inhibited how would cell function be affected? While the most recognised role of the MMPs is ECM degradation, their role in facilitating cellular access to stored growth factors is attracting greater research interest. Indeed, MMP-3 has been shown to release the large latent TGF-β_1 complex from the ECM prior to further proteolysis of the complex by other proteases such as MMP-2 and plasmin to release biologically active TGF-β_1 (Maeda *et al.* 2002).

Another point of interest was the recent report that TGF-β_1 and IGF-II could up regulate the expression and facilitate the function, respectively, of the IGFBP-4 protease pregnancy-associated plasma protein-A (PAPP-A) (a metalloproteinase). This resulted in irreversible liberation of IGFBP-4 bound IGF-I and subsequent enhanced growth of cultured human osteoblasts (Ortiz *et al.* 2003). IGFBP-4 has been demonstrated as inhibitory to IGF-I mediated cell responses in human osteoblast cells due to sequestration of IGF-I away from cultured cells (Mohan *et al.* 1995b). However, Kricker *et al.*, (2003) demonstrated that the 4 IGFBPs, including IGFBP-4 / IGF-I complexes, could be immobilised at tissue culture surfaces bound to VN (Kricker *et al.* 2003). Taken together, these data suggest that addition of VN to IGFBP-4 / IGF-I complexes could provide a useful mechanism by which a certain amount of IGF-I could be immobilised on a substrate seeded with cells and released at some later time by the addition of IGF-II and TGF-β_1.

1.9 BONE TISSUE ENGINEERING

Bone tissue engineering is a relatively recent approach to the long established field of skeletal engineering, or iatrogenic restoration, which has been practiced for many centuries in the repair of fractures and recently with the implantation of dental and orthopaedic devices. The development of this new approach offers the prospect of new therapies for clinically significant skeletal defects caused by developmental, pathologic or traumatic events. Bone tissue engineering, in general, encompasses a broad range of disciplines including cell and molecular biology, biochemistry, physiology, engineering, chemistry and medicine (Doll *et al.* 2001; Atala and Lanza 2002).

Over the past decade great emphasis has been placed on the development of cell based strategies for the repair of orthopaedic defects. In particular, technologies for the isolation and culture of bone marrow derived hMSCs have been developed (Haynesworth *et al.* 1992b). The *in vitro* expansion capability of these cells has been shown to exceed 1 billion fold (Bruder *et al.* 1997b). Osteoblasts are one of the end stage phenotypes of these cells and therefore are considered as a potential source of large numbers of cells which can be used in bioengineering of new bone tissue (Jaiswal *et al.* 1997). hMSCs are also capable of differentiating into chondrocytes,

myoblasts, stromal cells, fibroblasts and adipocytes (Caplan 2000). These cell types are responsible for the production and maintenance of cartilage, muscle, marrow stroma, tendon, ligament and connective tissue respectively. Entrance into and progression along individual lineage pathways for bone, cartilage, muscle and fat tissues can be stimulated by known inductive agents (Wakitani *et al.* 1995; Jaiswal *et al.* 1997; Johnstone *et al.* 1998; Pittenger *et al.* 1999). However, an area of continuing investigation is the molecular mechanism that governs each lineage pathway.

The population of hMSCs present in the marrow of the aged is considerably less than in the young, ~1:2,000,000 marrow cells compared to ~1:10,000 marrow cells respectively. As osteoblasts arise from hMSCs, this decrease in hMSC concentration in the aged may account for the observed decreased rates of bone repair. However, hMSCs can be mitotically expanded in culture and retain their ability to differentiate into the osteogenic lineage regardless of the donor's age (Ohgushi and Caplan 1999). This property of hMSCs, could enable aged patients to benefit from stem cell based bone tissue engineering therapeutics. However, a significant obstacle to the clinical use of *ex vivo* expanded stem cells of any type is that current culture methods require the use of animal derived additives to the culture media, commonly foetal bovine serum, potentially facilitating the transmission of pathogens to the patient. Moreover, serum contains a milieu of various proteins, cytokines, growth factors and ECM proteins, which can affect cell behaviour. Consequently, growth media used for classical tissue culture methods, where additives such as serum are used, are essentially undefined. Current regulatory trends find ill-defined reagents undesirable and therefore methods must be established which enable the *ex vivo* expansion of hMSCs for clinical use, in a highly defined growth media without animal derived products.

A great deal of progress has been made over the last decade developing novel biomaterials for use as implants and bone tissue substitutes for the repair of large orthopaedic defects. Some of these have been developed with a view to providing both a substrate for cell attachment and subsequent tissue in-growth and as a delivery vehicle for growth factors and cytokines to support normal bone tissue development and growth. Much effort has been directed toward development of materials which

exhibit both ‘osteoconductive’ and ‘osteoinductive’ properties. Osteoconductive materials are defined as being able to support bone in-growth and bone formation. Ceramics such as calcium phosphate, of which HA is the most common, and polymers such as collagens, have been shown to be effective conductors of bone formation. Osteoinductive materials stimulate *de novo* bone growth and can be classified as either physical or chemical stimulators. It is well documented that application of appropriate physical stress on bone augments bone tissue formation. Therefore, biomaterials, which mimic the mechanical properties of natural bone, and transmit normal physiological levels of stress to cells within the graft / implant or surrounding bone tissue, will assist the induction of bone formation and growth. Materials which contain chemical stimulators of bone formation are typically adsorbent materials able to bind biochemical elements such as ECM proteins, protein growth factors, other cytokines / hormones and / or drugs. Again, various calcium phosphates feature prominently in this category. There remains, however, significant knowledge gaps including identification of appropriate protein complexes which result in optimal bone growth and tissue formation. Logically, potential candidate proteins for inducement of bone tissue growth are those present in bone tissue itself. These include among others the IGFs, their associated binding proteins, particularly IGFBP-5, transforming growth factor-β_1 (TGF-β_1) and ECM proteins including, but not limited to, type I collagen, fibronectin and VN. Members of our laboratory have discovered novel interactions between VN and members of the IGF / IGFBP family which then elicit enhanced biological responses compared to any of the components in isolation (Upton *et al.* 1999; Kricker *et al.* 2003; Noble *et al.* 2003). In addition, TGF-β_1, also highly expressed in bone, has been found to interact with VN (Schoppet *et al.* 2002). Together these ECM protein / growth factor complexes may provide a way of expanding hMSCs in culture in the absence of animal derived products, while coincidently being able to finely control cell phenotype and function through the delivery of specific signals to the cell surface.

There are many studies in which MSCs and ECM proteins and / or growth factors have been combined with biomaterial matrices in animal models of connective tissue repair. These include MSC / collagen matrices for use in tendon repair (Young *et al.* 1998), hMSC / gelatin sponge composites in a rabbit model of cartilage regeneration (Ponticiello *et al.* 2000) and hMSC / porus hydroxyapatite / β-tricalcium phosphate

ceramic composites inserted in critical-sized femoral defects in athymic rats (Bruder *et al.* 1998b). In addition, MSCs have been shown to repair femoral defects in Spraugue-Dawley rats when injected into the gap in a rat femoral distraction model (Richards *et al.* 1999). Taken together, these studies demonstrate that the principle of stem-cell seeded scaffolds for bone tissue repair and regeneration has promise.

1.10 CONCLUSION

This review has outlined some of the major growth factors present in bone tissue and their common relationships with VN and FN. While little is known about whether these molecules (the IGFs, IGFBP-5, TGF-β_1, VN and FN) associate naturally in bone, it is apparent that these relationships could be important for *ex vivo*, animal product free culture of hMSCs for application in potential tissue engineered therapeutics. It is clear from the literature that there is a keen interest in the combined effects of TGF-β_1 and IGF-I on osteoblast function, bone formation and bone repair processes, especially in the context of tissue engineering (Blumenfeld *et al.* 2002a; Blumenfeld *et al.* 2002b; Schmidmaier *et al.* 2003; Wong *et al.* 2003). Equally, there is emerging evidence which suggests that proteases, especially the MMPs, play a direct role in regulation of bone cell function, such as cell proliferation and differentiation, by the extra-cellular processing of various growth factor complexes.

Through determination of the functional effects of and association between the above-mentioned molecules, it is my belief that more appropriate tissue culture methodologies can be developed that facilitate the clinical use of biomaterial / stem cell composite technologies for bone / connective tissue repair. Such studies will also help us to gain a greater understanding of the spatial relationships between growth factors, their binding proteins, the ECM / substrate and *in vitro* cultured bone cells. Utilising the knowledge and understanding gained so far in these convergent areas of study may also assist in the development of serum free culture methodologies for the *ex vivo* expansion of hMSCs for research and therapeutic purposes.

1.11 OUTLINE OF PROJECT

1.11.1 Hypothesis

The hypothesis tested by this thesis was that:

Specific combinations of the ECM protein VN and members of the IGF family of growth factors, such as IGFBP-3 or 5 and IGF-I, can support cell attachment, migration, proliferation and / or differentiation of human osteoblast like cells (SaOS-2) and hMSCs in culture in the absence of animal derived culture media additives.

1.11.2 Aims

Thus the specific aims of my PhD studies were to:

1) Determine the functional effect of various combinations of VN, IGFBP-5 and IGF-I on SaOS-2 cell migration, attachment and proliferation *in vitro*.
2) Determine the functional effect of various combinations of VN, IGFBP-3 or -5 and IGF-I or TGF-β_1 on hMSC *in vitro* expansion.
3) Determine the functional effect of various combinations of FN, IGFBP-5 and IGF-I on SaOS-2 cell migration and proliferation *in vitro*.
4) Determine the effect of selected combinations of ECM protein and growth factors on the expression of various osteogenic differentiation markers in SaOS-2 cells.

CHAPTER 2:

MATERIALS AND METHODS

2.1 MATERIALS

All general reagents were of the highest laboratory grade and were purchased from various companies.

2.1.1 Proteins

Purified human VN was purchased from Promega Corporation (Annandale, NSW, Australia). Recombinant human IGFBP-5, chicken anti IGFBP-5 polyclonal antibody and rabbit anti chicken-HRP conjugated 2° antibodies were produced as described previously (Baxter *et al.* 2002) and purchased from Dr Sue Firth (Kolling Institute of Medical Research, Sydney, NSW, Australia). IGFBP-3 N109D was purchased from Upstate (Waltham, MA, USA) while, IGF-I was purchased from GroPep Ltd (Adelaide, SA, Australia). Recombinant human TGF-β_1 and recombinant human bFGF was purchased from Chemicon Pty / Ltd (Boronia, Vic, Australia). Purified human FN was purchased from Sigma-Aldrich (St Louis, MO, USA) and recombinant human EGF was supplied by Invitrogen (Auckland, New Zealand).

2.1.2 Tissue culture

Tissue culture flasks and 96 well tissue culture plates were supplied by Nalge Nunc (Rochester, NY, USA) while 6 well tissue culture plates were supplied by Greiner Bio-one (Frickenhausen, Germany). Black CulturPlate™-96F tissue culture plates were supplied by PerkinElmer (Boston, MA, USA) and Costar® Transwells® were purchased from Corning COSTAR (New York, NY, USA). Dulbecco's Modified Eagle Medium (DMEM), Minimal Essential Medium, Alpha formulation (αMEM), Hanks Balanced Salt Solution (HBSS), penicillin / streptomycin, gentamycin and trypsin / EDTA were purchased from Invitrogen / GIBCO (Auckland, New Zealand). Foetal calf serum (FCS) was purchased from ThermoTrace (Noble Park, Vic, Australia) or Hyclone (Logan, UT, USA).

2.1.3 Experimental and general reagents

WST-1 reagent was purchased from Roche Applied Sciences (Brisbane, QLD, Australia). First Strand (AMV) cDNA synthesis kit, HEPES, Agarose, Collagenase B, DNase, 1-4-dithiolthetol (dTT) and Tris base were supplied by Roche Diagnostics

(Mannheim, Germany). Cyquant™ reagent was acquired from Molecular Probes / Invitrogen (Eugene, OR, USA). Crystal violet powder, Sigmacote® for coating plastic ware involved in preparation of VN or IGFBP solutions, Dexamethasone, Glycerol-2-phosphate, porcine gelatin, trypan blue, paraformaldehyde, diethylpyrocarbonate (DEPC) and Tri-Reagent were supplied by Sigma-Aldrich (St Louis, MO, USA). Fraction V RIA grade bovine serum albumin were obtained from Sigma-Aldrich (St Louis, MO, USA) or Calbiochem (San Diego, CA, USA). L-Ascorbic acid 2-phosphate trisodium salt was purchased from Wako chemical Ltd (Osaka, Japan). SYBR® green PCR master mix was purchased from Applied Biosystems (Warrington, UK). ECL Plus™ western blot detection system was supplied by Amersham Biosciences (Little Chalfont, Buckinghamshire, UK). Magnesium chloride, Gycerol, Triton X100, D-Glucose, potassium chloride, chloroform, propane-2-ol (isopropanol), methanol and ethanol were acquired from BDH Laboratory Supplies (Poole, UK). Pre-cast 4-20% Tris-HCl gradient gels and gelatin zymograms were run on gel electrophoresis equipment obtained from BIORAD Laboratories Pty / Ltd (Regents Park, NSW, Australia). Coomasie G250, TEMED and 40% acrylamide / Bis were also supplied by BIORAD Laboratories. Tween-20 was obtained from Research Organics (Cleveland, OH, USA) while phosphate buffered saline (PBS) was purchased from Oxoid (Basingstoke, Hampshire, England).

2.2 CELLS AND CULTURE CONDITIONS

2.2.1 SaOS-2 cells

Human osteoblast like, SaOS-2 cells, originally isolated from a human osteosarcoma, were obtained from the American Type Culture Collection (Rockville, Maryland, USA; HTB-85). The cells were maintained in αMEM (Gibco) supplemented with 10% FCS (ThermoTrace or Hyclone) 50 U/mL penicillin / streptomycin (Gibco) and 5 µg/mL gentamycin (Gibco) (αMEM+) and were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C. Culture media was changed twice weekly and sub-cultivated prior to confluence. Cultures were discarded after 20 passages to ensure the phenotype of the cell population was maintained.

2.2.2 Human bone marrow derived mesenchymal stem cells (hMSCs)

Bone marrow samples were collected with hospital and university ethics committee approvals and informed consent from both female and male patients presenting for total knee or hip joint replacement surgery for treatment of osteoarthritis at either The Prince Charles Hospital or The Holy Spirit Northside Hospital in Brisbane. Typically 2-5 mL of bone marrow was collected during surgery by the surgeon and placed into tubes containing 5 mL PBS supplemented with 200 U/mL heparin and placed on ice for transport to QUT. Collagenase B (0.02%) and DNase (100 U/mL) were occasionally employed to assist in the break up of any clots, otherwise the marrow suspension was repeatedly passed through a 19 gauge needle to dislodge clumped cells. The samples were then diluted to 30 mL with PBS, thoroughly mixed and passed through a 100 µm filter to remove extracellular debris and bone fragments. Following overlaying of 30 mL of the filtrate onto 15 mL of Lymphoprep™ the samples were centrifuged at 400 g for 35 min at 20°C with zero brake. The mononuclear cell fraction (buffy coat) was removed, placed into a fresh tube and with 20 mL DMEM (Gibco) supplemented with 10% FCS (ThermoTrace), 50 U/mL penicillin/streptomycin (Gibco) and 5 µg/mL gentamycin (Gibco) ($DMEM^+$), then centrifuged at 1000 rpm for 10 min. The pellet was resuspended in 1 mL of $DMEM^+$ (cells for culture) or serum-free DMEM (sf-DMEM) (for CFU-F assays) prior to counting with a haemocytometer. Cells were then seeded into tissue culture flasks at 1.6 x 10^5 cells/cm^2 and incubated in a humidified 5% CO_2/95% air atmosphere at 37°C. Culture media ($DMEM^+$) was changed after 5 days in the first instance, then twice weekly thereafter. At passages 4-6 each patient's cells were characterised for their multilineage (osteogenic, chondrogenic and adipogenic) potential by myself and / or others within our team using standard histochemical, bioactivity and marker expression assays relavent to each of the lineages (Yameen *et al.* 2007).

2.3 PRE-BINDING OF VITRONECTIN, FIBRONECTIN, IGFBP-5 AND IGF-I TO TISSUE CULTURE WELLS

SaOS-2 cell attachment and proliferation were assessed on pre-bound combinations of vitronectin (VN) (Promega), fibronectin (FN)(Sigma-Aldrich), IGFBP-5 (Dr Sue Firth) and IGF-I (Gropep). Specifically, 52 µL of 1 µg/mL VN / sf-αMEM, 6 µg/mL

FN / sf-αMEM or sf-αMEM alone was added to each well of a 96 well tissue culture plate (Nalge Nunc) or a black 96F tissue culture plate (PerkinElmer) and incubated for 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation, the ± VN / sf-αMEM solution was removed and each well was washed with 174 µL of freshly made 0.5% BSA / Hepes Binding Buffer (HBB) (0.1 M Hepes, 0.12 M NaCl, 5 mM KCl, 1.2 mM $MgSO_4 \cdot 7H_2O$, 8 mM D-Glucose, pH 7.6). Wells were then blocked with 44 µL of freshly made 1% BSA / HBB and incubated for 30 mins in the above conditions, the blocking solution was then removed and each well washed with 174 µL 0.5% BSA / HBB and 43.5 µL of IGFBP-5 (1.6 µg/mL) in 0.5% BSA / HBB, either alone or in combination with IGF-I (0.4 µg/mL), were added to wells with or without VN and incubated at 4°C overnight.

2.4 PRE-BINDING OF VITRONECTIN, FIBRONECTIN, IGFBP-5 AND IGF-I TO TRANSWELLS™

SaOS-2 cell migration was assessed on pre-bound combinations of VN or FN, IGFBP-5 and IGF-I. Specifically, 1 mL of 1 µg/mL VN / sf-αMEM, 6 µg/mL FN / sf-αMEM or sf-αMEM alone was added to the lower chamber of each well of 12 well Transwell™ tissue culture plates (Costar) and incubated for 4 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation, the ± VN or FN / sf-αMEM solutions were removed and each well was washed twice with 1 mL of freshly made 0.5% BSA / HBB. Subsequently, duplicate 0.5 mL aliquots of IGFBP-5 (0.1 µg/mL) in 0.05% BSA / HBB /sf-αMEM together with either 0.5 mL IGF-I (0.025 µg/mL) in 0.05% BSA / HBB / sf-αMEM or 0.5 ml of 0.05% BSA / HBB / sf-αMEM was added to wells with or without VN and incubated at 4°C overnight. Following incubation (during cell preparation), the growth factor solutions were aspirated and the lower chambers washed twice with 1 mL 0.05% BSA / HBB / sf-αMEM followed by the addition of 1 mL 0.05% BSA / HBB / sf-αMEM to the lower chamber of each Transwell™ and returned to the incubator until required.

2.5 PREPARATION OF SOLUTION PHASE VN AND GROWTH FACTORS FOR hMSC STUDIES.

hMSC cell metabolic activity and change in total protein were assessed in the presence of solution phase combinations of VN, IGFBP-3 or -5 and IGF-I or TGF-β_1. Specifically, 52 µL of 1 µg/mL (52 ng/well) or 3.346 µg/mL (174 ng/well) VN / serum-free-DMEM (sf-DMEM) solution or sf-DMEM alone was added to each well of 96 well tissue culture plates (Nalge Nunc) and incubated for 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation, 43.5 µL of 0.16 µg/mL (7 ng/well) TGF-β_1 or 1.6 µg/mL (70 ng/well) IGFBP-3 or -5 in 0.5% BSA / sf-DMEM either alone or in combination with 0.4 µg/mL (17.4 ng/well) IGF-I were added to wells with or without VN and incubated at 4°C overnight. Plates were then allowed to return to room temperature prior to seeding of cells. Final concentrations of added proteins following seeding of cells (final volume of 195.5 µL) were 0.266 µg/mL (3.55 nM) or 0.890 µg/mL (11.9 nM) (VN), 0.358 µg/mL (11.9 nM) (IGFBPs), 89 ng/mL (11.9 nM) (IGF-I) and 36 ng/mL (1.43 nM) (TGF-β_1).

2.6 MIGRATION ASSAYS

SaOS-2 cell migration through the microporus membranes of 12 µm pore Transwells™ was measured using a modified version of the Transwell™ migration assay described previously by (Leavesley *et al.* 1992; Leavesley *et al.* 1993). Briefly, sub-confluent cultures of SaOS-2 cells were passaged 1:1 the day prior to assay and incubated over night in a humidified 5% CO_2/95% air atmosphere at 37°C. Cultures were then washed once with HBSS for 5 min and serum starved for 4 hr in sf-αMEM to minimise the influence of residual serum proteins in the assay without adversely affecting cell viability. Cells were then washed again with HBSS and harvested with 1 mL of 0.05% trypsin / EDTA solution. The cells were suspended in sf-αMEM, centrifuged; the pellet resuspended in fresh 0.05% BSA / HBB / sf-αMEM and the cells were counted prior to dilution to 5 x 10^5 cells/mL. Previously prepared Transwell™ plates were removed from the incubator and the upper chamber of each Transwell™ were seeded with 2 x 10^5 cells and were then allowed to incubate for 5 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells seeded into wells without VN were employed as controls. Following incubation, the Transwell™ plates were placed on ice to reduce further migration. Transwell™

inserts were individually removed from the plates and un-migrated cells removed from the upper surface of the insert by cleaning with a cotton bud for approximately 1.5 min/insert as previously described by (Leavesley *et al.* 1993) and J. Kricker (Kricker 2005). Migrated cells were then fixed by immersing the membranes in formaldehyde for 20 min and subsequently stained by immersing the membranes in crystal violet for 20 min. Inserts were then immersed in a beaker of circulating tap water to remove the excess stain and were allowed to air dry prior to extraction of crystal violet in 1 mL of 10% acetic acid. Duplicate 100 µL sub-samples were then transferred to individual wells of a 96 well microtitre plate and the absorbance read in a BioRad Bench Mark Plus microplate reader (BIORAD Laboratories Pty / Ltd (Regents Park, NSW, Australia) at 595 nm. Results are expressed as the absorbance as a percentage above the VN only control ± the standard error of the means (SEM).

2.7 ATTACHMENT ASSAY

SaOS-2 cell attachment was measured in cultures grown for 3 hr on each treatment by spectrophotometric detection of the cleavage product (Formazan) of the tetrazolium salt (4-[3-(4-Iodophenyl)-2-(4nitrophenyl)-2H-5-tetrazolio]1,3-benzene disulfonate) (WST-1) (Roche Diagnostics). Briefly, sub-confluent cultures of SaOS-2 cells were washed twice with Hanks Balanced Salt Solution (HBSS) (Gibco) for 5 min and serum starved for 4 hr in sf-αMEM. Cells were then washed again with HBSS and harvested with 1 mL of 0.05% trypsin / EDTA solution. The cells were suspended in sf-αMEM, centrifuged, the pellet resuspended in fresh sf-αMEM and cells counted prior to dilution to 5 x 10^4 cells/mL. Each well received 5 x 10^3 cells suspended in 100 µL of sf-αMEM and were allowed to incubate for 3 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells seeded into wells without VN, or cells seeded in normal growth media containing 10% FCS, were employed as controls. Following incubation, the media was removed and each well was washed once with sf-αMEM prior to addition of 110 µL of 1:10 dilution of WST-1: sf-αMEM to each well, and incubated for a further 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation the plates were placed in a Beckman microplate reader (Beckman, Inc. Fullerton, USA) and the absorbance of the formazan dye cleavage product quantitated at 450 nm using 650 nm as reference. Results are from 3 separate experiments with treatments performed in at least

triplicate per experiment and are expressed as the corrected absorbance at 450 nm - 650 nm as a percentage of the +VN control ± standard error of the means (SEM).

2.8 CyQUANT® CELL PROLIFERATION ASSAY

SaOS-2 cell number was measured in cultures grown for 24 hr, 48 hr or 72 hr on each treatment by fluorescent detection of cellular nucleic acids using CyQUANT® GR nucleic acid binding dye (Molecular Probes). Briefly, sub-confluent cultures of SaOS-2 cells were washed twice with HBSS (Gibco) for 5 min and serum starved for 4 hr in sf-αMEM. The cells were then washed with HBSS and harvested with 1 mL of 0.05% trypsin / EDTA solution. The cells were suspended in sf-αMEM, centrifuged at 1000 rpm for 6 min, the pellet resuspended in fresh sf-αMEM and the cells counted prior to dilution to 5 x 10^4 cells/mL. Each well of the pre-prepared black 96F plates (as detailed above) received 5 x 10^3 cells suspended in 100 µL of sf-αMEM and were allowed to incubate for 24 hr, 48 hr or 72 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells seeded into wells without VN or FN, or cells seeded in normal growth media containing 10% FCS, were employed as controls. For the purposes of constructing a cell number standard curve, 1 x 10^6 cells were added to a conical tube, centrifuged at 1000 rpm for 6 min, the supernatant was removed and the pellet stored at -80°C until required for analysis. Following incubation, the spent media was removed from each well and the plates were wrapped in parafilm and stored at -80°C until analysis with CyQUANT® GR dye. On the day of analysis fresh CyQUANT® GR reagent was prepared as per the manufacturers instructions. Briefly, a 1X CyQUANT® GR working solution was prepared in foil-covered 50 mL polypropylene conical tubes by addition of ddH_2O, kit component B (20X concentrated lysis buffer) and kit component A (400X CyQUANT® GR stock solution) and thorough mixing. A cell number standard curve was prepared by firstly adding 1 mL of CyQUANT® GR working solution to the pellet of cells kept from the time of seeding of the assay, followed by breaking of the cell pellet and cells by trituration and vortexing. The resulting suspension thus represented the total nucleic acid content of 1 x 10^6 cells. Serial dilutions made to correspond to cell numbers ranging from 50 cells to 50,000 cells in 200 µL volumes were added in triplicate to a black 96F microtitre plate. CyQUANT® GR working solution alone served as a “0 cells” control. Each treatment well of the black 96F

plates received 200 µL of CyQUANT® GR working solution and was incubated for ~ 15 min, (protected from light) prior to measurement of the sample fluorescence at 480 nm excitation and 520 nm emission using a POLARstar OPTIMA fluorescence micro-plate reader (BMG Labtech, Offenburg, Germany). The raw data were corrected with plate blanks (CyQUANT® GR working solution alone), converted to cell number and the results expressed as the mean cell number ± SEM. Data were pooled from at least triplicate samples and the experiments were repeated at least 3 times.

2.9 METABOLIC ACTIVITY ASSAY

SaOS-2 or hMSC metabolic activity was measured in cultures grown for 48 hr (SaOS-2) or 24 hr, 48 hr and 72 hr (hMSCs) on each treatment by spectrophotometric detection of the cleavage product (Formazan) of the tetrazolium salt WST-1 (Roche Diagnostics). Briefly, sub-confluent cultures of SaOS-2 cells were washed twice with HBSS (Gibco) for 5 min and serum starved for 4 hr in sf-αMEM. SaOS-2 cells or hMSCs were then washed with HBSS and harvested with 1 mL of 0.05% trypsin / EDTA solution. The cells were suspended in sf-αMEM (SaOS-2 cells) or sf-DMEM (hMSCs), centrifuged, the pellet resuspended in fresh sf-media and cells counted prior to dilution to 5 x 10^4 cells/mL. Each well received 5 x 10^3 cells suspended in 100 µL of respective sf-media and were allowed to incubate for 48 hr (SaOS-2) or 24 hr, 48 hr or 72 hr (hMSCs) in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells seeded into wells without VN, or cells seeded in normal growth media containing 10% FCS, were employed as controls. Following incubation, 10 µL of WST-1 reagent was added to each well (SaOS-2 cells) or the media was removed and 110 µL of 1:10 dilution of WST-1:sf-DMEM was added to each well (hMSCs), and incubated for a further 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation the plates were placed in a BioRad Bench Mark Plus microplate reader (BIORAD Laboratories Pty / Ltd (Regents Park, NSW, Australia) and the absorbance of the formazan dye cleavage product quantitated at 450 nm using 650 nm as reference. Results are from 3 (SaOS-2 cells) or 4 (hMSCs) separate experiments performed in at least triplicate and are expressed as the corrected absorbance at 450 nm - 650 nm as a percentage of the +VN control (SaOS-

2 cells) or as a percentage of the 24 hr –VN control ± standard error of the means (SEM).

2.10 COLONY FORMING UNIT-FIBROBLASTIC ASSAY

Plate preparation for colony forming unit fibroblastic assay included pre-binding of VN and growth factors similar to that described in section 2.3 or leaving the growth factors in solution. The colony forming unit assays were performed in 6 well plates (Costar). While the growth factor concentrations were kept the same as described in section 2.3 the VN concentration was increased. Specifically, 1.4 mL of 4 µg/mL (5.6 µg/well) VN / sf-DMEM solution was added to each well of a 6 well tissue culture plate and incubated for 2.5 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation the VN solution was removed and 1.4 mL of 0.16 µg/mL (224 ng/well) TGF-β_1 or 1.6 µg/mL (2.24 µg/well) IGFBP-3 or -5 in 0.5% BSA / HBB either alone or in combination with 0.4 µg/mL (560 ng/well) IGF-I were added to wells with VN and incubated at 4°C overnight. Growth factor solutions were then removed prior to seeding of freshly harvested bone marrow mononuclear cells at a density of 1.6 x 10^5 cells/cm^2 in a final volume of 3 mL/well (3.4 mL/well solution phase studies). For solution phase studies the VN was allowed to pre-bind as described above, however, the growth factors were left in solution upon seeding of cells. Therefore the final growth factor concentrations for the solution phase studies were 0.066 µg/mL (2.6 nM) TGF-β_1 or 0.659 µg/mL (22 nM) IGFBP-3 or -5 either alone or in combination with 0.164 µg/mL (22 nM) IGF-I. hMSCs exposed to un-treated wells or to 10% FCS served as controls. Cells were allowed to attach and grow for up to 7 days during which each culture was observed using a Nikon TS100 inverted phase contrast microscope (NIKON, Tokyo, Japan) and digital photographic images taken on days 1, 3 or 4 and day 7 using a microscope mounted NIKON COOLPIX 4500 digital camera (NIKON).

2.11 TOTAL PROTEIN ASSAY

hMSCs were harvested by trypsin digestion prior to seeding of 5000 cells/well into 96 well plates containing various combinations of VN, IGFBP-5 and IGF-I in solution as detailed in section 2.5. Briefly, sub-confluent cultures of hMSCs were washed twice with HBSS and harvested with 1 mL of 0.05% trypsin / EDTA

solution. The cells were suspended in sf-DMEM, centrifuged at 1000 rpm for 6 min and the pellet resuspended in fresh sf-DMEM prior to performing cell counts using a haemocytometer and trypan blue and subsequent dilution to 5 x 10^4 cells/mL. Each well received $5x10^3$ cells suspended in 100 µL of sf-DMEM and was allowed to incubate for 24 hr, 48 hr or 72 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Conditioned media was then removed for protease activity analysis (see section 2.13 & 2.14) and monolayers were fixed by addition of 50 µL of 4% paraformaldehyde solution to each well and incubated for 20 min at room temperature. The fixative was removed prior to the addition of 50 µL of freshly prepared crystal violet and incubated at room temperature for a further 20 min. The crystal violet was then removed and the plates immersed in a beaker of gently circulating tap water until the stain had visibly ceased washing from wells. Plates were allowed to air dry overnight, after which the bound crystal violet stain was solubilised with 100 µL of 10% acetic acid per well and mixed by gentle vibration. Each plate was placed in a BioRad Bench Mark Plus microplate reader (BIORAD Laboratories Pty / Ltd (Regents Park, NSW, Australia) and the absorbance readings taken at 595 nm. Results are from 4 separate experiments performed in at least triplicate and are expressed as the corrected absorbance at 595 nm as a percentage of the 24 hr -VN control ± standard error of the means (SEM).

2.12 MORPHOLOGICAL ANALYSIS

Where stated digital photographic images depicting culture morphology were taken at X100 or X200 magnification using a NIKON TS100 inverted, phase contrast microscope (NIKON, Tokyo, Japan) and a microscope mounted NIKON COOLPIX 4500 digital camera (NIKON). Scale was determined using a stage micrometre (PYSER-SGI, Edenbridge, Kent, UK).

2.13 GELATIN ZYMOGRAPHY

Conditioned media was removed from representative wells from the total protein assays (section 2.11) immediately prior to fixing of cells with paraformaldehyde and were stored at -20°C until required for analysis. Samples were then analysed for MMP-2 and MMP-9 gelatinolytic activity. Briefly, 4 µL of 5X loading buffer (0.05% bromophenol blue, 50% glycerol, 10% SDS, 0.5 M Tris base, pH6.8) was

added to 16 µL of conditioned media samples and the samples were loaded onto freshly prepared 4% stacking / 10% resolving polyacrylamide gels containing 1 mg/mL gelatin and subjected to electrophoresis in Tris-glycine running buffer (25 mM Tris base, 246 mM glycine, 0.1% SDS, pH 6.8) under non-reducing conditions at 200 volts and 4°C until the dye front had just run off the gel. The gels were then washed in 2.5% Triton-X100 for 30 min and then again for 60 min to remove SDS and incubated in incubation buffer (50mM Tris base, 10mM $CaCl_2$, 50mM NaCl, pH 7.6) for 48 hr at 37°C. The gels were stained with 0.25% Coomasie Blue (4.5:4.5:1 – water: methanol: acetic acid) and subsequently destained with 10% acetic acid and 40% methanol (vol / vol). Gelatinase activity was visualised by the presence of clear bands and discriminated according to molecular weight and comparison to pro-MMP-2 and pro-MMP-9 standards (Chemicon).

2.14 IGFBP-5 DEGRADATION ASSAY

The conditioned media from hMSCs cultured in sf-DMEM alone was collected and stored as above (section 2.11) prior to testing for the ability to degrade IGFBP-5. Briefly, 20 µL samples of phosphate buffered saline, sf-DMEM or hMSC conditioned media were mixed with 2 µL of 200 ng/µL IGFBP-5 (400 ng) (Dr Sue Firth, Kolling Institute of Medical Research) or vehicle and were incubated overnight at 37°C. Samples were pulsed centrifuged followed by the addition of 5 µL of 50 mM 1-4-dithiolthetol (dTT) to 20 µL of each sample and were subsequently heated at 95°C for 15 min. Samples were then pulse centrifuged and 12 µL of each sample was subsequently run on a 4%-20% gradient polyacrylamide gel (BIORAD) in Tris-glycine buffer (25 mM Tris base, 200 mM glycine, 0.1% SDS, pH 8.3) at 100 volts for 1 hr at 4°C. Proteins were then transferred to a BioTrace® NT nitrocellulose membrane (Pall Corporation, Pensacola, FL, USA) in transfer buffer composed of 25 mM Tris base, 40 mM Glycine, 10% methanol. The nitrocellulose membrane was subsequently blocked with 3%BSA (w / v) in TBST (10 mM Tris base, 140 mM NaCl, 1% Tween 20) for ~2 hr and was then washed in TBST for 30 min, with the buffer replaced every 5 min. The bound proteins were then interrogated with a 1:1000 dilution of a chicken anti IGFBP-5 polyclonal antibody (Dr Sue Firth) in 0.3% BSA / TBST at 4°C over night. The membrane was then washed for 30 min, with the buffer replaced every 5 min and subsequent 1°

antibody detection with a 1:10,000 dilution of a rabbit anti chicken-HRP conjugated 2° antibody (Dr Sue Firth) in 0.3% BSA / TBST at room temperature for 40-45 min. The membrane was washed in TBST for 30 min with the buffer replaced every 5 min prior to the detection of the 2° antibody by incubation of the membrane for 2 min in 2 mL of chemiluminescent substrate prepared as per manufacturers instructions (ECL Plus Western Blot detection kit)(Amersham). Chemiluminescence was then captured by brief exposure of the membrane to x-ray film (Agfa) and subsequently developed using an Agfa automated film developer CP-1000 (Mortsel, Belgium).

2.15 SOLUTION PHASE CULTURE OF SAOS-2 CELLS

Solutions of VN / sf-αMEM (1 µg/mL) or FN / sf-αMEM (6 µg/mL) or VN / FN / sf-αMEM (1 µg/mL and 6 µg/mL for VN and FN respectively) were added to individual wells of a 6 well tissue culture plate (Greiner) together with either EGF (5 ng/mL) or bFGF (5 ng/mL). SaOS-2 cells were harvested as detailed below (section 2.16) prior to addition of 1.44 x 10^5 cells/well and incubation in a humidified 5% CO_2/95% air atmosphere at 37°C. Media was changed twice weekly using protein concentrations detailed above. Digital images were captured at various time points throughout the culture period as indicated in the figures and detailed in section 2.12.

2.16 DIFFERENTIATION ASSAY CULTURES

SaOS-2 cell osteoblast marker expression was assessed on the following pre-bound combinations of proteins: VN alone; VN / IGFBP-5 / IGF-I; VN / TGF-β_1 and VN / FN / EGF. Specifically, 1.5 mL of a 13.3 nM solution of VN / sf-αMEM (1 µg/mL) or FN / sf-αMEM (6 µg/mL) or VN / FN / sf-αMEM (1 µg/mL / 6 µg/mL) were added to individual wells of a 6 well tissue culture plate (Greiner) and incubated for 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation, VN ± FN sf-αMEM solutions were removed and each well was washed with 1.5 mL of freshly made 0.5% BSA / HBB. Wells were then blocked with 1.26 mL of freshly made 1% BSA / HBB and incubated for 30 mins in the above conditions after which, the blocking solution was removed and each well washed again with 1.5 mL 0.5% BSA / HBB. Specific wells then received 1.263 mL of sf-αMEM with either 5.34 nM IGFBP-5 / IGF-I (160 ng/mL / 40 ng/mL for IGFBP-5 and IGF-I respectively),

4.61 nM TGF-β_1 (115.2 ng/mL) or 17.7 nM EGF (115.2 ng/mL) or sf-αMEM alone. Plates were then sealed with parafilm and incubated at 4°C overnight. Growth factor solutions were then aspirated and each well then washed with 1.5 mL sf-αMEM prior to the addition of 1.5 mL of sf-αMEM to treatment wells, 10% FCS or osteogenic supplemented media (+OS media) (100 nM Dexamethasone, 50 µM Ascorbic acid-2-phosphate, 10 mM β-glycerophosphate, sf-αMEM) to control wells. Sub-confluent cultures of SaOS-2 cells were washed twice with HBSS prior to harvest by Trysin / EDTA digestion and re-suspension in sf-αMEM. Cells were counted and seeded at 5 x 10^5 cells per well in a total volume of 3 mL. Plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 hr or for 7 days (with 1 media change for the 7 day experiment on day 4 using the same growth factor concentrations as described above). Digital photographs were taken to assess culture morphology prior to RNA extraction or on other days as stated.

2.17 REALTIME PCR ANALYSIS

Real time PCR analysis was performed to determine if VN alone, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF could induce osteoblast marker expression in SaOS-2 cells. The spent media from differentiation assay cultures at either 48 hr or 7 days was aspirated and the remaining cell monolayer harvested using a rubber cell scraper and 1 mL per well of Tri-Reagent (Sigma-Aldrich). The cell suspension was then transferred to sterile 2 mL eppendorf tubes, immediately placed on ice and subsequently stored at -80°C until required for RNA extraction. RNA extraction was performed as per the Tri-Reagent RNA extraction protocol (Sigma-Aldrich). Briefly, following thawing to room temperature, 0.2 mL of chloroform was added to each sample followed by gentle shaking for 15-20 sec. Samples were then allowed to stand at room temperature for 10 min and subsequently centrifuged at 12,000 x g for 15 minutes at 4°C. The colourless upper aqueous phase was carefully transferred to individual fresh 2 mL eppendorf tubes followed by addition of 0.5 mL of 100% isopropanol and gentle mixing. Samples were then allowed to stand at room temperature for 10 min prior to further centrifugation at 12,000 x g for 10 min at 4°C. The supernatant was carefully removed and the RNA pellets washed with 1 mL 75% ethanol followed by gentle mixing and centrifugation at 7500 x g for 5 min at 4°C. The supernatant was removed and the pellets were allowed to air dry to remove

excess ethanol. Each pellet was resuspended in 20 µL of diethylpyrocarbonate (DEPC) treated ddH_20, then transferred to fresh 0.5 mL eppendorf tubes and immediately placed on ice. RNA integrity was ascertained by subjecting 1 µL of each sample, together with 9 µL ddH_20 and 2 µL loading dye, to gel electrophoresis through a 1.5% agarose gel containing 0.01% ethidium bromide. Gels were run for ~25 min at 106 volts in TBE buffer prior to image capture in a Syngene UV transilluminator cabinet using GeneSnap image software. RNA concentration and quality of each sample was determined by subjecting 2 µL of total RNA and 78 µL of TE buffer to UV spectrophotometry using a GeneQuant II UV spectrophotometer (Pharmacia Biotech, Cambridge, UK). RNA samples were subsequently stored at -80°C until required for cDNA synthesis.

cDNA synthesis was performed on each total RNA sample using a First Strand cDNA synthesis kit (AMV) according to manufacturers instructions (Roche). Briefly, 0.5 µg of total RNA was added to a reaction mixture containing 10 mM Tris, 50 mM KCl, 5 mM $MgCL_2$, 1 mM dNTPs (dATPs, dCTPs, dTTPs, dGTPs 1 mM each), 2.0 µL (3.2 µg) random hexamers, 1.0 µL (50 units) RNase inhibitor, 0.8 µL (≥ 20 units) AMV reverse transcriptase and ddH_20 to a final volume of 20 µL in 0.2 mL PCR tubes. Each cDNA synthesis reaction tube was gently mixed, pulse centrifuged and incubated in a PTC-200 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA) at 25°C for 10 min, 42°C for 60 min, 99°C for 5 min and finally 4°C overnight. The cDNA samples were then stored at -80°C until required for real time-PCR analysis. Real time-PCR was carried out using an ABI Prism® 7000 real time-PCR machine (Applied Biosystems, Foster City, CA, USA) and Axygen PCR microplates (Axygen Scientific, Union City, CA, USA) were utilised for sample reactions. Immediately prior to real time-PCR analysis each cDNA sample was diluted 1:5 with TE buffer. Two µL of diluted cDNA template was then added in triplicate to individual PCR reaction mixtures containing 10 µL of SYBR® green PCR master mix (Applied Biosystems), 5 pM of forward primer, 5 pM of reverse primer and ddH_20 to a final volume of 20 µL per well. The loaded PCR microplates were pulse centrifuged, placed into the ABI Prism 7000 real time-PCR machine and subjected to the following cycle protocol: 50°C for 2 min, 95.0°C for 10 min then 40 cycles of 95.0°C for 15 sec and 60.0°C for 1 min followed by a dissociation stage of 95.0°C for 15 sec, 60°C for 20 sec and 95°C for 15 sec. Raw

data was exported to Microsoft Excel, transformed and analysed using the $2^{-\Delta\Delta CT}$ method according to User Bulletin # 2 for ABI Prism® 7000 Sequence Detection System and Livak and Schmittgen (Livak and Schmittgen 2001). cDNA prepared as above from cells just prior to seeding (un-seeded cells) was utilised as a time 0 control to which all treatments were compared. The raw data was normalised against the expression of 18s ribosomal RNA expression levels and the results are expressed as mean fold change compared to the time 0 (un-seeded) cell control ± SD from triplicate PCR reactions for each of 3 separate differentiation experiments. PCR primer sequences targeting the osteoblast marker genes transcript cDNA for core binding factor-1 (cbfa-1), alkaline phosphatase (ALP) and type 1 collagen (COL-1) are presented in table 2.1. (Sequences for cbfa-1 were kindly shared by Shea Carter, ALP and COL-1 primers were kindly donated by Anna Coussens and 18s sequences were kindly shared by Dr Steven Myers).

Table 2.1 Real time-PCR Primer Sequences

Target	Primer Sequence
cbfa -1	Forward 5′-TGATGACACTGCCACCTCTGA-3′ Reverse 5′-AAAGGTGGCTGGATAGTGCAT-3′
ALP	Forward 5′-CGTGGCTAAGAATGTCATCATGTT-3′ Reverse 5′-TGGTGGAGCTGACCCTTGA-3′
COL-1	Forward 5′-CGAAGACATCCCACCAATCAC-3′ Reverse 5′-TTGTCGCAGACGCAGATCC-3′
18s	Forward 5′-TTCGGAACTGAGGCCATGAT-3′ Reverse 5′-CGAACCTCCGACTTTCGTTC-3′

2.18 STATISTICAL ANALYSIS

Results are expressed as the pooled means as a percentage of either the negative control or of VN alone as indicated ± the standard error of the means (SEM). In some instances the results are expressed as the pooled means of cell number ± SEM as indicated. Differences between groups were analysed by post-hoc t-test generally or post-hoc Dunnet's test for the attachment assay in Chapter 3, and significance was accepted at $p<0.05$. Experiments were repeated at least 3 times unless otherwise stated in the experimental procedures section of each results chapter.

CHAPTER 3:

EFFECTS OF PRE-BOUND VITRONECTIN, IGFBP-5 AND IGF-I ON OSTEOBLAST LIKE SAOS-2 CELL MIGRATION, ATTACHMENT, METABOLIC ACTIVITY AND PROLIFERATION

3.1 INTRODUCTION

The IGFs (IGF-I and IGF-II) are 2 of the most abundant growth factors present in the bone matrix (Mohan and Baylink 1996). Osteoblasts (cells which lay down new bone matrix) are known to express large quantities of the IGFs (Canalis *et al.* 1988) which they secrete along with IGF binding proteins (IGFBPs), including IGFBP-5 (Andress and Birnbaum 1992). IGFBP-5 is thought to be responsible for securing the IGFs within the bone matrix due to its ECM and hydroxyapatite (the mineral component of bone tissue) binding capabilities (Bautista *et al.* 1991). IGFBP-5 has also been shown to bind to a wide range of ECM proteins such as type III and IV collagens, laminin (Jones *et al.* 1993), osteopontin, thrombospondin (Nam *et al.* 2000), fibronectin (Jones *et al.* 1993; Xu *et al.* 2004) and importantly, vitronectin (Nam *et al.* 2002; Kricker *et al.* 2003). IGF-I is known to have stimulatory effects on osteoblast proliferation both *in vivo* and *in vitro* (Andress and Birnbaum 1992). Similarly IGFBP-5 has been associated with the stimulation of cell proliferation in general (Andress and Birnbaum 1992; Miyakoshi *et al.* 2001; Schneider *et al.* 2002). However, IGF-I has also been associated with the progression of osteoblast differentiation processes (Jia and Heersche 2000). This is of interest because generally when pre-osteoblast cultures begin to differentiate the rate of proliferation decreases (Stein and Lian 1993). This suggests that IGF-I may not have a role in initiating these processes but rather, may act as a driving mechanism for differentiation.

VN, is a 75 kDa, multifunctional glycoprotein found in blood plasma and in the ECM (Bale *et al.* 1989; Seiffert 1996; Seiffert *et al.* 1996a; Seiffert *et al.* 1996b; Seiffert and Schleef 1996; Schvartz *et al.* 1999). VN is synthesised by osteoblasts during bone development and remodelling and is a significant component of non collagenous bone ECM (Seiffert 1996; Kumagai *et al.* 1998; Schvartz *et al.* 1999). VN is thought to play a role in facilitating cellular processes during bone remodelling and was recently found to influence osteoblastic differentiation (Salasznyk *et al.* 2004). VN contains an RGD sequence which enables integrin-binding (Felding-Habermann and Cheresh 1993; Schvartz *et al.* 1999), and both osteoblasts and osteoclasts express $\alpha_v\beta_3$ integrins which facilitate cell attachment to

various ECM proteins including VN (Pytela *et al.* 1985; Koistinen *et al.* 1999; Cheng *et al.* 2001; Kilpadi *et al.* 2004; Lai and Cheng 2005).

In 1999, Upton *et al*. reported that insulin like growth factor II was able to bind directly to VN, but also found that IGF-I could not bind directly to VN (Upton *et al.* 1999). IGFBP-5 has since been found to bind to VN with high affinity and via this interaction was able to modulate IGF-I actions *in vitro* (Nam *et al.* 2002; Kricker *et al.* 2003). In particular, IGF-I mediated migration and DNA synthesis in porcine aortic smooth muscle cells is modulated (Nam *et al.* 2002). It has also been demonstrated that pre-bound combinations of VN, IGFBP-5 and IGF-I can facilitate protein synthesis and migration in both a human keratinocyte cell line (HaCAT) and primary human keratinocytes, as well as enhance migration in MCF-7 breast cancer cells (Kricker *et al.* 2003; Noble *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005).

The functional responses that have been observed in epithelial tissue types in response to pre-bound combinations of VN, IGFBP-5 and IGF-I, along with the prevalence of these factors in bone tissue, and the important role they play in osteoblast function, suggested that these factors may mediate similar responses in mesenchymal type cells such as osteoblasts. Furthermore, many modern strategies for the development of advanced connective tissue therapeutics are focusing on implantable constructs consisting of a biomaterial scaffold, incorporating some form of ECM with incorporated growth factors and/or *ex vivo* expanded undifferentiated cells (Goshima *et al.* 1991; Dennis and Caplan 1993; Wakitani *et al.* 1995; Bruder *et al.* 1997; Jaiswal *et al.* 1997; Bruder *et al.* 1998a; Bruder *et al.* 1998b; Caplan *et al.* 1998; Johnstone *et al.* 1998; Pittenger 1999; Noshi *et al.* 2000; Caplan and Bruder 2001; Shimaoka *et al.* 2004). This approach is designed to deliver a large number of precursor cells, which subsequently differentiate into appropriate tissue types through interactions with both the accompanying scaffold / ECM and the surrounding host tissue.

Based on the previous findings within our research team, and the growing interest in ECM/growth factor complexes for use in bone tissue therapeutics, I decided to investigate the efficacy of combinations of VN, IGFBP-5 and IGF-I as a pre-bound stimulus for bone cell function. In particular, these studies focussed on exploring the

hypothesis that pre-bound combinations of VN, IGFBP-5, and IGF-I would mediate migration, attachment and proliferation responses in osteoblasts. In order to test this hypothesis the osteoblast-like cell line SaOS-2 was selected as they have been extensively characterised in the literature (Murray *et al.* 1987; Rodan *et al.* 1987; Fedde *et al.* 1988; Farley *et al.* 1991; Mayr-Wohlfart *et al.* 2001; Schmid *et al.* 2001; Kilpadi *et al.* 2004; Pautke *et al.* 2004), they are responsive to IGF-I (Schmid *et al.* 1999; Schmid *et al.* 2001) and attach to VN (Degasne 1999; Kilpadi *et al.* 2004). In addition, they have been widely used for investigations involving the IGF system and interactions with various ECM proteins (Mohan *et al.* 1993; Kudo *et al.* 1996; Kudo *et al.* 1997; Degasne 1999; Schmid *et al.* 1999; Bostedt *et al.* 2001; Schmid *et al.* 2001; Kilpadi *et al.* 2004). The following experiments were designed to assess SaOS-2 osteoblast function, namely: 1) cell migration; 2) cell attachment; 3) cellular metabolic activity; and 4) cell proliferation in response to various pre-bound combinations of VN, IGFBP-5 and IGF-I.

3.2 EXPERIMENTAL PROCEDURES

Full details of both the materials and methods used in the generation of the data presented in this chapter are described in chapter 2. The following are brief summaries of the materials and experimental procedures used for the generation of data presented in section 3.3.

3.2.1 Materials

Purified human VN was purchased from Promega Corporation. IGFBP-5 was produced as described previously by Firth et al. (1999) and purchased from Dr Sue Firth, while IGF-I was purchased from GroPep Ltd. Costar® Transwells® were purchased from Corning Corporation. WST-1 reagent was purchased from Roche Applied Science. Cyquant™ reagent was purchased from Molecular Probes. Standard 96 well tissue culture plates were supplied by Nalge Nunc. Black 96F tissue culture plates were supplied by Perkin Elmer, while fraction V RIA grade Bovine serum albumin was supplied by Sigma or Calbiochem. Sigmacote® for coating of plastic-ware used in the preparation of VN or IGFBP-5 solutions and crystal violet powder for use in migration assays was purchased from Sigma.

3.2.2 Pre-binding of proteins

For full details of this method please refer to section 2.3 and 2.4. SaOS-2 cell migration was assessed on pre-bound combinations of VN, IGFBP-5 and IGF-I. Briefly, the underside of Transwell™ tissue culture plate inserts (Costar) were coated with 1 mL of 6 μg/mL VN / sf-αMEM or sf-αMEM alone for 4 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation, the cells were washed 2 times with 1 mL of 0.5% BSA / HBB. Duplicate, 0.5 mL aliquots of IGFBP-5 (0.1 μg/mL) in 0.05% BSA/HBB / sf-αMEM, together with either 0.5 mL IGF-I (0.025 μg/mL) in 0.05% BSA / HBB / sf-αMEM or 0.5 ml of 0.05% BSA / HBB / sf-αMEM, was added to wells with or without VN and incubated at 4°C overnight. Following incubation the lower chambers were washed twice with 1 mL 0.05% BSA / HBB / sf-αMEM prior to the addition of 1 mL 0.05% BSA / HBB / sf-αMEM to the lower chamber of each Transwell™ and incubation until required.

SaOS-2 cell attachment, proliferation and metabolic activity were assessed on pre-bound combinations of VN, IGFBP-5 and IGF-I. Specifically, 52 μL of a 1 μg/mL VN / sf-αMEM solution or sf-αMEM alone was added to each well of a 96 well tissue culture plate and incubated for 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following removal of +/-VN / sf-αMEM solution, wells were washed with 0.5% BSA/HBB. Wells were then blocked with 1% BSA / HBB, incubated for 30 min in the above conditions and then each well washed with 0.5% BSA / HBB followed by addition of 43.5 μL of IGFBP-5 (1.6 μg/mL) in 0.5% BSA / HBB either alone or in combination with IGF-I (0.4 μg/mL), and with or without VN. The plates were incubated at 4°C overnight.

3.2.3 Transwell™ Migration Assay

For full details of this method please refer to section 2.6. Sub-confluent cultures of SaOS-2 cells were passaged 1:1 the day prior to assay and serum starved for 4 hrs before harvesting and seeding 2 x 10^5 cells into the upper chamber of 12 μm pore Transwells™ which had been pre-coated with various combinations of VN, IGFBP-5 and / or IGF-I. Following 5 hr incubation, un-migrated cells were removed from the upper surface of the Transwell™ membrane with a cotton bud. Migrated cells were fixed in formaldehyde and stained with crystal violet. Excess stain was

removed and the membranes were allowed to air dry prior to extraction of crystal violet dye with 10% acetic acid. Sub-samples were transferred to a 96-well microtitre plate and the absorbance read at 595 nm. Results are from 2 (-VN treatments) or 3 (+VN treatments) separate experiments with each treatment tested in triplicate and are expressed as the mean absorbance as a percentage above the +VN control ± the standard error of the mean (SEM).

3.2.4 Attachment Assay

For full details of this method please refer to section 2.7. Sub-confluent cultures of SaOS-2 cells were serum-starved for 4 hr prior to harvest and seeding of 5000 cells/well into 96-well plates containing various pre-bound combinations of VN, IGFBP-5 and IGF-I. Cells were allowed to attach for 3 hr prior to washing of wells to remove unattached cells and subsequent treatment of each well with WST-1 / αMEM solution. Absorbance readings at 450 nm - 650 nm were taken following a 2 hr incubation. Results are from 2 separate experiments performed in at least quintuplicate and are expressed as the corrected absorbance at 450 nm-650 nm as a percentage of the +VN control ± standard error of the means (SEM).

3.2.5 Proliferation Assay

For full details of this method please refer to section 2.8. Sub-confluent cultures of SaOS-2 cells were serum-starved for 4 hr prior to harvest and seeding of 5000 cells/well into black 96-well plates containing various pre-bound combinations of VN, IGFBP-5 and IGF-I. Cells were allowed to proliferate for 24 hr, 48 hr or 72 hr prior to removal of the spent media and storage of the culture plates at -80°C until analysis. Cyquant™ reagent was added to each well and the fluorescence measured at 480 nm excitation and 520 nm emission. Fluorescence units were converted to cell number by using cells kept from the time of seeding to construct a cell number standard curve as per the manufacturer's instructions. Results are from 3 separate experiments and are expressed as the mean cell number ± standard error of the means (SEM).

3.2.6 Metabolic Activity Assay

For full details of this method please refer to section 2.9. Sub-confluent cultures of SaOS-2 cells were serum starved for 4 hr prior to harvest and seeding of 5000 cells/well into 96-well plates containing various pre-bound combinations of VN, IGFBP-5 and IGF-I. Cells were allowed to grow for 48 hr prior to addition of WST-1 reagent. Absorbance readings at 450 nm - 650 nm were taken following a 2 hr incubation. Results are from 3 separate experiments and are expressed as the corrected absorbance at 450 nm - 650 nm as a percentage of the +VN control ± standard error of the means (SEM).

3.3 RESULTS

3.3.1 Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell migration

VN is widely known to facilitate the migration of a variety of cell types (Leavesley *et al.* 1992). Indeed members of our laboratory have previously demonstrated that pre-bound combinations of VN / IGFBP / IGF-I enhance MCF-7 cell migration through Transwells™ with 12 μm pore membranes (Kricker *et al.* 2003). Therefore I performed Transwell™ migration assays in order to assess whether or not pre-bound combinations of VN / IGFBP-5 / IGF-I stimulated a migration response in the osteoblast-like, osteosarcoma cell line, SaOS-2 (Figure 3.1).

SaOS-2 cell migration was significantly enhanced in the presence of VN ($p<0.01$, n=9) over treatments without VN (n=6). Indeed, in the absence of VN, responses were less than 13% ± 2.6% of the +VN control, irrespective of subsequent IGFBP-5 and / or IGF-I treatment. When either IGFBP-5 or IGF-I were pre-bound to VN coated Transwells™, cell migration responses were 96.7 ± 8.7% and 126.8 ± 9.9% of the +VN control respectively. Statistical analysis of these data using post-hoc t-test revealed that there was no significant difference between either of these values and the +VN control. However, this analysis revealed that there was a significant difference between the VN / IGFBP-5 response and the VN / IGF-I response ($p<0.05$). When IGFBP-5 and IGF-I were both pre-bound to VN-coated Transwells™ the migratory response was 198.1 ± 6.8% of the +VN control and was significantly higher than any other treatment ($p<0.01$). The significant increase in cell migration response to treatments containing pre-bound VN suggests that the

presence of VN was vital to support the migration of SaOS-2 cells through the membranes. In addition, the results obtained for the VN / IGFBP-5 / IGF-I treatment reflect a synergistic effect on the cell migration response of SaOS-2 cells.

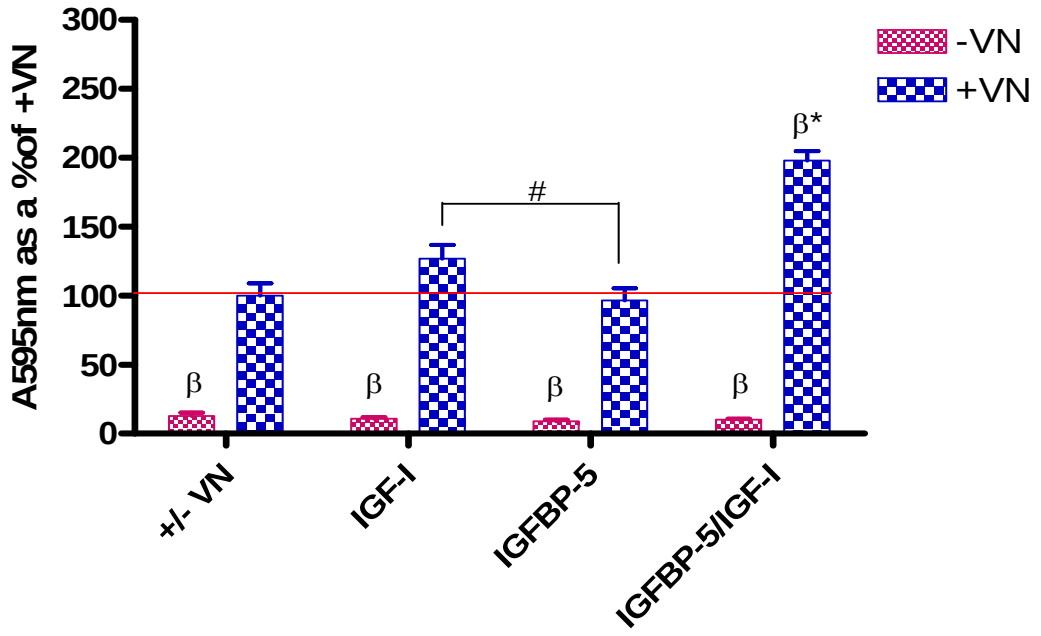


Figure 3.1. Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell migration. SaOS-2 cells (2 x 10^5 cells/Transwell™) were incubated in sf-αMEM and allowed to migrate for 5 hr on pre-bound combinations of IGFBP-5 (50 ng/well) and IGF-I (12.5 ng/well) in the presence or absence of pre-bound VN (1 µg/well). Following incubation, un-migrated cells were removed from the upper surface of the Transwell™ by cleaning with a cotton bud for 1.5 minutes/insert. Migrated cells were then fixed in formaldehyde for 20 min and subsequently stained with crystal violet for 20 min. Excess stain was removed by immersion of the inserts in a reservoir of circulating tap water and air dried prior to extraction of crystal violet in 0.5 mL of 10% acetic acid. Triplicate 100 µL sub-samples were then transferred to individual wells of a 96-well micro-titre plate and the absorbance read at 595 nm. Results were corrected using data from plate blanks (10% acetic acid alone) and are expressed as the corrected absorbance as a % of the VN only control (100%) ± standard error of the means (SEM). Significant difference to the VN only control is indicated by β (p<0.01) and was determined by post-hoc t-test. Significant difference to all other groups or select groups (where linked by line) is indicated by # (p<0.05) or * (p<0.01).

3.3.2 Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell attachment

VN is well known for mediation of cell attachment *in vitro* via interaction with its cell surface receptors the α_v integrins, in particular the $\alpha_v\beta_3$ integrin. In view of this attachment assays were performed in order to determine the effects of pre-bound combinations of VN / IGFBP-5 / IGF-I on SaOS-2 cell attachment. As expected, SaOS-2 cells exhibited significantly enhanced attachment to treatments where pre-bound VN was present ($p<0.01$, n=11) compared to wells without VN (n=11)(Figure 3.2). Post-hoc Dunnet's test, was used in this instance in order to compare the effects of single treatments to baseline conditions ie +VN. The results obtained for treatments where VN was not present were similar to the background levels indicating that after 3 hr essentially no cells had been able to attach to the culture surface of the wells. When IGF-I, IGFBP-5 or both were pre-bound to wells containing pre-bound VN or in the presence of 10% FCS, values obtained for SaOS-2 cell attachment were 45.5 ± 21.4%, 39.9 ± 11.5%, 38.4 ±18.0% or 20.7 ± 15.5% respectively above the +VN control. However, statistical analysis of these data indicated that the increase was not significant. Observation of the cultures supported the quantitative data, as cells cultured in the presence of VN or 10% FCS appeared to be firmly attached and had well spread morphologies. In contrast, cells seeded into wells without VN or FCS appeared weakly attached and had rounded morphologies following the 3 hr incubation time period and prior to wash steps (not shown). These results indicate that, within this system, VN is a vital component for facilitating SaOS-2 cell attachment to the tissue culture surface and neither IGF-I nor IGFBP-5 together, or in isolation, have a significant effect.

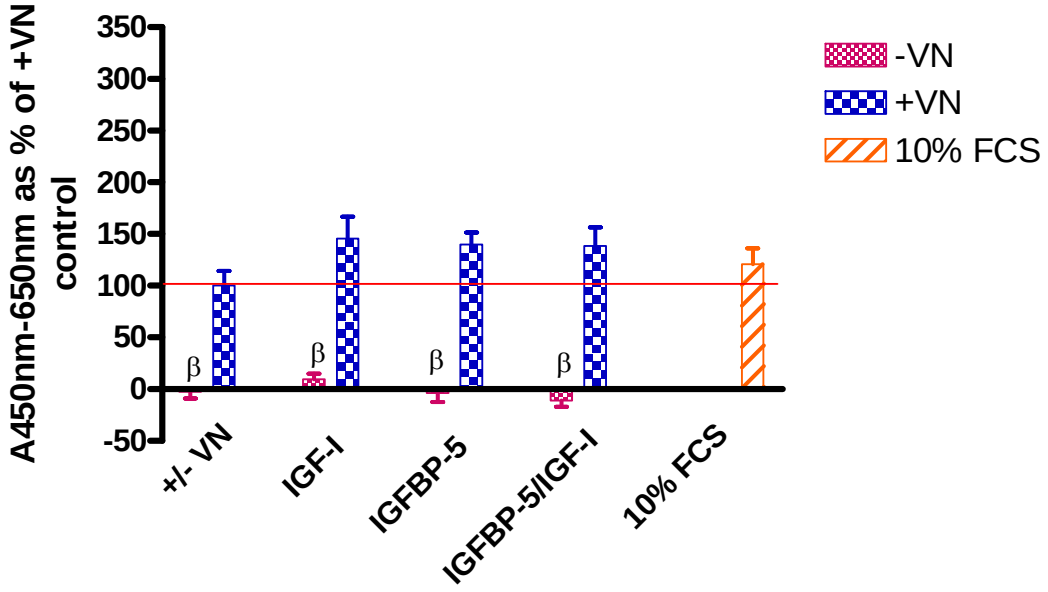


Figure 3.2. Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell attachment. SaOS-2 cells were incubated in sf-αMEM and allowed to attach for 3 hr on pre-bound combinations of IGFBP-5 (70 ng/well) and IGF-I (17 ng/well) in the presence or absence of pre-bound VN (52 ng/well). The media was then removed and the cells were washed once with sf-αMEM prior to addition of 110 µL of 1:10 dilution of WST-1 reagent / sf-αMEM and incubated for a further 2 hr. Cells seeded into wells without VN, or with normal growth media containing 10% FCS were employed as controls. The absorbance of the formazan dye cleavage product was then quantitated at 450 nm using 650 nm as reference. Results were corrected using data from plate blanks (serum free media and WST-1 alone) and are expressed as the corrected absorbance at 450 nm - 650 nm ± standard error of the means (SEM). Significant difference between individual treatments and the +VN control was determined by post-hoc Dunnet's test and is indicated by α (p<0.05) or β (p<0.01).

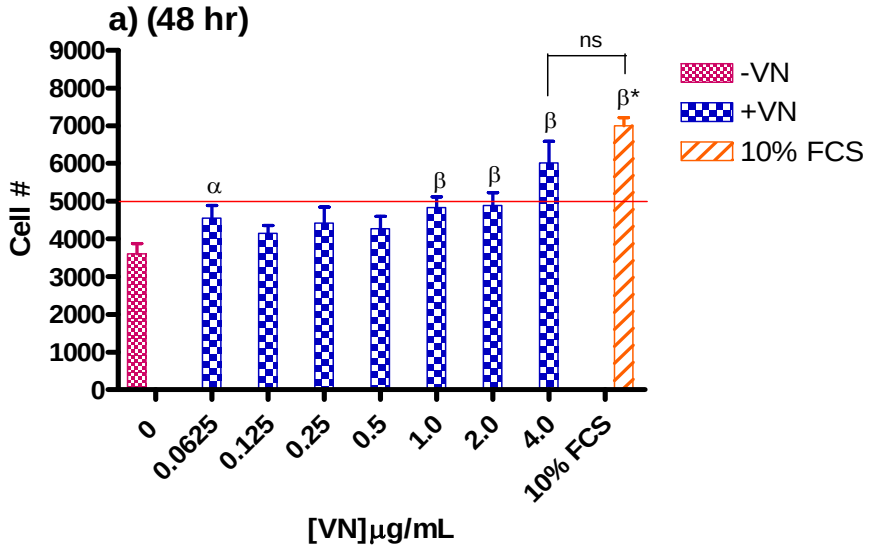
3.3.3 Effect of various doses of pre-bound VN on SaOS-2 cell proliferation.

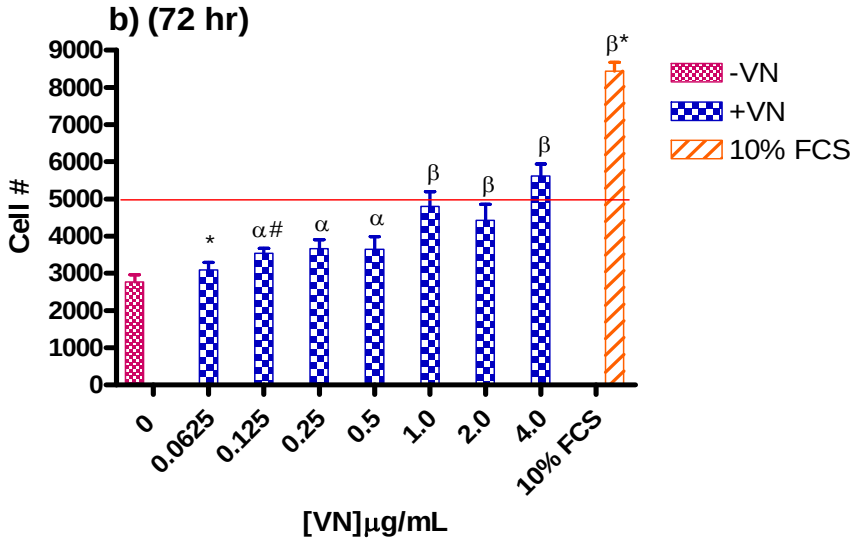
In order to determine if VN alone has an effect on SaOS-2 cell proliferation, a dose response time course was performed (Figure 3.3). Cell number for each of the VN concentrations, 10% FCS and the –VN control were not significantly different after 24 hr and had not increased above the initial cell seeding density of 5000 cells (for simplicity select data for the 24 hr time point is shown in Figure 3.3c). At 48 hr the 10% FCS control had increased to 6999 ± 212 cells (n=15), which was not significantly more than the cell density observed with 4 μg/mL of VN (6012 ± 328 cells, n=6) but was significant compared to the 2 μg/mL (4887 ± 340 cells, n=13), 1 μg/mL (4831 ± 288 cells, n=15), 0.5 μg/mL (4268 ± 332 cells, n=9), 0.25 μg/mL (4420 ± 426 cells, n=9), 0.125 μg/mL (4152 ± 210 cells, n=9) or 0.0625 μg/mL (4548 ± 338 cells) VN treatments, as well as to the –VN control (3606 ± 278 cells, n=15) ($p<0.01$) (Figure 3.3a). Cell number in the 4 μg/mL, 2 μg/mL and 1 μg/mL VN concentrations were all significantly higher than the –VN control ($p<0.01$). However, there was no statistical difference between these treatments; indeed they had not substantially changed from the initial number of cells seeded (5000 cells/well). Interestingly, cell numbers in the 0.0625 μg/mL VN treatment were also found to be significantly higher than the –VN control ($p<0.05$), although significance was only just attained.

By 72 hr, however, the 10% FCS control had increased to 8442 ± 231 cells (n=12). This is significantly greater than the number of cells observed for the 4 μg/mL (5618 ± 328 cells, n=6), 2 μg/mL (4418 ± 435 cells, n=12), 1 μg/mL (4802 ± 389 cells, n=12), 0.5 μg/mL (3645 ± 339 cells, n=6), 0.25 μg/mL (3665 ± 237 cells, n=6), 0.125 μg/mL (3536 ± 136 cells, n=6) or 0.0625 μg/mL (3083 ± 203 cells, n=6) VN treatments and the –VN control (2767 ± 193 cells, n=12) (*p*0.01) (Figure 3.3b). Since 1 μg/mL is the standard VN concentration used in assays with other cell types (Kricker *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005), I also determined the statistical significance between this concentration and the various other VN concentrations evaluated. Again there was no statistical difference between the 4 μg/mL, 2 μg/mL or 1 μg/mL VN concentrations at 72 hr; indeed, there had been no significant increase or decrease in cell number between 24 hr and 72 hr in these 3 treatments. There was also no statistical difference between the 1 μg/mL treatment and the 0.25 μg/mL or 0.5 μg/mL VN treatments, and although cell numbers had

decreased between 48 hr and 72 hr in these 2 treatments the decrease was not significant. A 24hr time point was not measured for VN concentrations below 1 μg/mL, however, there was a general trend for a decrease in cell number in treatments below 1 μg/mL over the 72 hr period. However, it is likely that the decrease would be significantly less than the initial number of cells seeded. As expected, the –VN control resulted in a steady decrease in cell number over 72 hr to 2767 ± 193 cells, which was significantly less than at 24 hr (4053 ± 312 cells) (p<0.01) and significantly less than all VN treatments above 0.125 μg/mL at 72 hr (p<0.01). Taken together the results described here and shown in figure 3.3 indicate that VN alone is able to sustain SaOS-2 cell survival for up to 72 hr in a dose dependent manor within the range of doses tested.

Figure 3.3. Effect of various doses of pre-bound VN on SaOS-2 cell proliferation. SaOS-2 cells (5000/well) were incubated in sf-αMEM and allowed to proliferate for various times, (a) 48 hr; b) 72 hr; and c) select time course data, in wells pre-coated with 50 µL solutions of VN at various concentrations as indicated. Cells seeded into wells without VN or in 10% FCS were employed as controls. Following incubation, the media was aspirated and the tissue culture plates were stored at -80°C. Cyquant reagent was then added to each well as per the manufacturer's instructions and the fluorescence measured at 480 nm excitation and 520 nm emission. Results were corrected using data from plate blanks (Cyquant reagent only) and cell number calculated from a cell number standard curve and expressed as mean cell number ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significance between treatments and the –VN control is indicated by α ($p<0.05$) or β ($p<0.01$). Significance between the 1 µg/mL treatment and all other treatments is indicated by # ($p<0.05$) or * ($p<0.01$), while ns indicates no significant difference between select data.


a) (48 hr)
Cell #
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
ns
α
β
β*
-VN
+VN
10% FCS
0
0.0625
0.125
0.25
0.5
1.0
2.0
4.0
10% FCS
[VN]μg/mL


b) (72 hr)
Cell #
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
β*
β
β
β
α
α#
α
*
-VN
+VN
10% FCS
0
0.0625
0.125
0.25
0.5
1.0
2.0
4.0
10% FCS
[VN]μg/mL

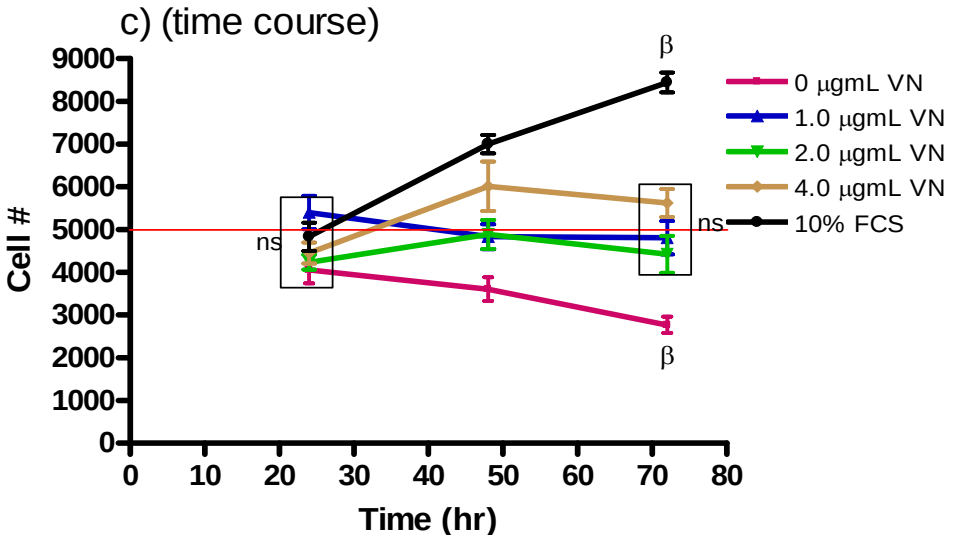

c) (time course)
Cell #
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
β
β
ns
ns
0 μgmL VN
1.0 μgmL VN
2.0 μgmL VN
4.0 μgmL VN
10% FCS
0
10
20
30
40
50
60
70
80
Time (hr)

Figure 3.3

3.3.4 Effect of various doses of pre-bound IGFBP-5 and IGF-I on SaOS-2 cell proliferation.

In order to determine if IGFBP-5 / IGF-I complexes have a dose-response effect on SaOS-2 cell proliferation, dose response assays were performed over 72 hr (Figure 3.4). Increasing amounts of IGFBP-5 and IGF-I in stoichiometrically equivalent ratios were added to tissue culture wells coated with 1 μg/mL VN (50 ng/well) or un-coated wells. Similar to the previous results, the -VN control significantly decreased in cell number from the initial seeding density of 5000 cells/well to 2407 ± 171 cells/well after 72 hr ($p<0.01$, n=9) (Figure 3.4). While increasing the IGFBP-5 / IGF-I dose in the absence of VN had a significant dose effect on increasing cell number compared to the –VN control, higher cell numbers were not attained until the 230 ng/mL IGFBP-5 / 57.6 ng/mL IGF-I dose (3321 ± 200 cells/well, n=9) ($p<0.01$). However, there was no further increase in cell number despite a 4 fold increase in IGFBP-5 / IGF-I to 920 ng/mL / 230.4 ng/ml. The overall response in the absence of VN reflected significant cell loss over 72 hr. Conversely, a dose-dependent increase was observed with increasing dose of pre-bound IGFBP-5 / IGF-I in the presence of pre-bound VN. Specifically, significantly higher cell number was reached in the 115 ng/mL IGFBP-5 / 28.8 ng/mL IGF-I treatment (6741 ± 256 cells/well, n=9) ($p<0.01$) compared to the +VN control (5312 ± 269 cells/well, n=9), whereas lower doses were found to not be significantly different to the +VN control. Similar to the higher IGFBP-5 / IGF-I doses without VN, the maximum significant increase in cell number over 72 hr in the presence of VN was reached with the 115 ng/mL IGFBP-5 / 28.8 ng/mL IGF-I treatment, with the higher doses not significantly increasing the cell number after 72 hr of culture. The largest mean increase, however, was observed for the highest IGFBP-5 / IGF-I dose (6909 ± 390 cells/well, n=9). Nevertheless, as stated above, this was not significantly different from the 115 ng/mL IGFBP-5 / 28.8 ng/mL IGF-I treatment. All the same it is worth noting that this represents approximately 70% of the response found with the 10% FCS control (9855 ± 332 cells/well, n=9).

The inset in Figure 3.4 shows time course data for the 115 ng/mL / 28.8 ng/well, IGFBP-5 / IGF-I dose in the presence of VN and indicate increases in cell number between 24 hr (n=9) and 72 hr (n=9), thus demonstrating cell proliferation (Figure 3.4 inset).

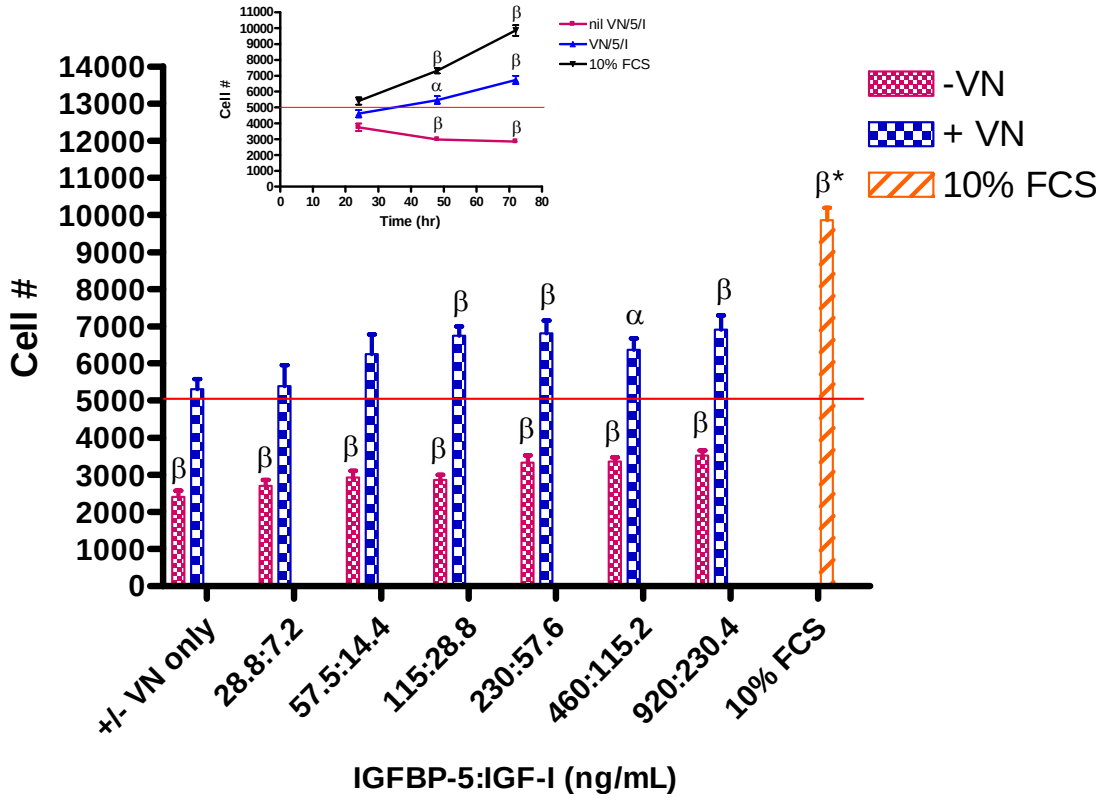


Figure 3.4. Effect of various doses of pre-bound IGFBP-5 and IGF-I on SaOS-2 cell proliferation. SaOS-2 cells (5000/well) were incubated in serum free-αMEM and allowed to proliferate for up to 72 hr in wells pre-coated with solutions of IGFBP-5 and IGF-I at various concentrations (as indicated) in the presence or absence of pre-bound VN (1 µg/mL). Cells seeded into wells without VN or in 10% FCS were employed as controls. Following incubation, media was aspirated and the tissue culture plates were stored at -80°C. Cyquant reagent was then added to each well as per the manufacturer's instructions and the fluorescence measured at 480 nm excitation and 520 nm emission. Results were corrected using data from plate blanks (Cyquant reagent only) and cell number calculated from a cell number standard curve and expressed as mean cell number ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significant difference to the +VN control is indicated by α (p<0.05) or β (p<0.01). Significant difference to all other groups is indicated by * (p<0.01). Horizontal red line indicates initial number of cells seeded per well. Inset shows the increase in cell number over time in the 115 ng/mL IGFBP-5 / 28.8 ng/mL IGF-I treatment in the presence (VN/5/I) or absence (nil VN/5/I) of VN, thus demonstrating cell proliferation. Significant difference to the 24 hr value for each treatment is indicated by α (p<0.05) and β (p<0.01).

3.3.5 Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell metabolic activity

A series of assays for metabolic activity were performed to determine the viability of individual cultures on pre-bound combinations of VN, IGFBP-5 and IGF-I after 48 hr of culture. In general, SaOS-2 cell metabolic activity was significantly higher (post-hoc t-test) for all treatments in the presence of VN (n=16) compared to those treatments without VN (n=16) (p<0.01) (Figure 3.5). Specifically, SaOS-2 cells cultured in the presence of VN / IGFBP-5 / IGF-I had significantly higher metabolic activity (227.2 ± 11.6%) than that of the +VN control (100 ± 1.5%), VN / IGF-I (124.2 ± 3.7%), and VN / IGFBP-5 (133.4 ± 5.7%) (p<0.01) treatments but was equivalent to that measured for cells cultured in 10% FCS (262.5 ± 37.6%). While, no statistical difference was found between the results obtained for cells cultured in the presence of VN / IGF-I and VN / IGFBP-5, both of these treatments significantly enhanced the cell metabolic activity over the +VN control (p<0.01). The same trends were observed for those treatments without VN. Specifically, cells exposed to IGFBP-5 / IGF-I had significantly higher metabolic activity (110.2 ± 8.1%) than that of the -VN control (27.8 ± 2.0%), IGF-I only (44.4 ± 2.0%), and IGFBP-5 only (45.1 ± 2.5%) (p<0.01) treatments. No statistical difference was found between the results obtained for cells cultured in the presence of IGF-I only, and IGFBP-5 only; however, both of these treatments significantly enhanced cell metabolic activity over the -VN control (p<0.01). Also, there was no difference between the results for the +VN control and IGFBP-5 / IGF-I, suggesting that IGFBP-5 was able to bind to the tissue culture surface, retain IGF-I and sustain biological activity. Interestingly, adding the results of the +VN control and IGFBP-5 / IGF-I, following correction of each by subtraction of the –VN control value, gives a value significantly less (154.7 ± 11.8) than the corrected VN / IGFBP-5 / IGF-I value (199.4 ± 13.6) (p<0.05) (Figure 3.5 inset). This clearly indicates that the combination of VN, IGFBP-5 and IGF-I has a synergistic effect on SaOS-2 cell metabolic activity over 48 hr. It is not clear however whether or not the increased metabolic activity is due to an increase in the number of metabolically active cells or due to an increase in metabolic activity of individual cells.

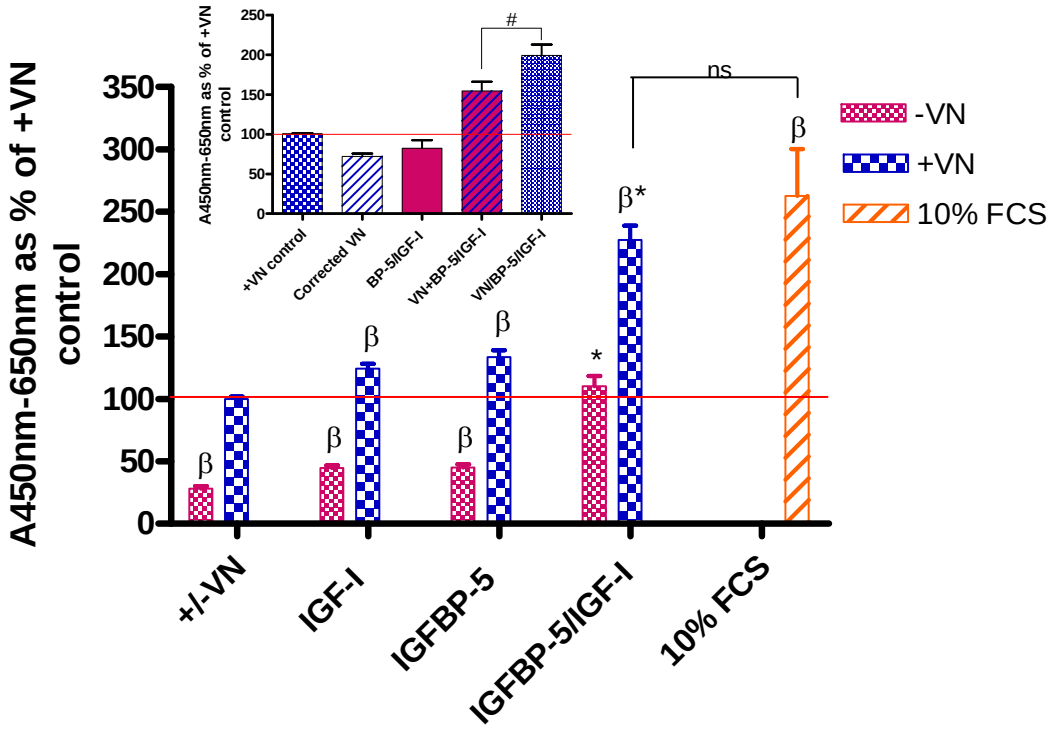


Figure 3.5. Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell metabolic activity. SaOS-2 cells were incubated in serum free-αMEM and allowed to proliferate for 48 hr on pre-bound combinations of IGFBP-5 (69.5 ng/well) and IGF-I (17.4 ng/well) in the presence or absence of pre-bound VN (52.1 ng/well). Following incubation, 10 µL of WST-1 reagent was added to each well and incubated for a further 2 hr. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. The absorbance of the formazan dye cleavage product was then quantitated at 450 nm using 650 nm as reference. Results were corrected using data from plate blanks (serum free media and WST-1 alone) and are expressed as the corrected absorbance at 450 nm - 650 nm ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significant difference to the +VN control is indicated by β ($p<0.01$). Significant difference between IGFBP-5 / IGF-I groups (± VN) and the individual components alone (± VN) is indicated by * ($p<0.01$). Inset shows the VN (▨) and IGFBP-5 / IGF-I (■) values following correction of each by subtraction of the -VN control. The additive effect of these corrected values (▩) is compared to the corrected value for the VN / IGFBP-5 / IGF-I treatment (▨). Significance between the 2 values is indicated by # ($p<0.05$). Horizontal red line indicates +VN control (100%).

3.3.6 Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell proliferation.

I wished to determine the effects that various combinations of VN, IGFBP-5 and IGF-I had on SaOS-2 cell proliferation and to determine if the observed increase in metabolism observed after 48 hr (Fig 3.5) was due to an increase in the number of metabolically active cells. I therefore performed a series of cell proliferation assays to determine the cell number at 24 hr intervals up to 72 hr (Figure 3.6a, b, c, n=18 for all treatments unless otherwise specified). After 24 hr only the 10% FCS control (5577 ± 123 cells/well) had increased in cell number above the initial cell seeding density of 5000 cells/well. In contrast, the +VN control was unchanged (4927 ± 154 cells/well) (p<0.01) (Figure 3.6a), yet was significantly higher than the –VN control (3948 ± 206 cells/well)(p<0.01), IGF-I only (4027 ± 174 cells/well)(p<0.01), IGFBP-5 only (3587 ± 235)(p<0.01) and VN/IGFBP-5 (4415 ± 180 cells/well) (p<0.05) treatments. Statistical analysis indicated there was no significant difference between the results obtained for VN / IGF-I (4733 ± 150 cells/well), IGFBP-5 / IGF-I (4810 ± 258 cells/well), VN / IGFBP-5 / IGF-I (4691 ± 199 cells/well) and the +VN control. However, the VN / IGFBP-5 treatment (4415 ± 180 cells/well) had significantly less cells than the +VN control after 24 hr.

At 48 hr all treatments exposed to pre-bound VN had increased above the initial cell seeding density (Figure 3.6b). In particular, increased cell number between the 24 hr time point and the 48 hr time point were recorded for the + VN control (5589 ± 174 cells/well), VN / IGF-I (5754 ± 179 cells/well), VN / IGFBP-5 (5312 ± 210 cells/well), VN / IGFBP-5 / IGF-I (5935 ± 185 cells/well) and 10% FCS (7248 ± 286 cells/well) treatments. While there was no statistical significance between any of the groups exposed to pre-bound VN and the +VN control, a significant difference in cell number existed between the VN / IGFBP-5 / IGF-I treatment and the VN / IGFBP-5 treatment (p<0.05). Cell numbers in the 10% FCS control at 48 hr were significantly higher than any other group (p<0.01). Cell numbers declined between 24 hr and 48hr in the –VN control (3123 ± 108 cells/well), IGF-I only (3558 ± 154 cells/well), IGFBP-5 only (3137 ± 133 cells/well) and IGFBP-5 / IGF-I (4560 ± 130 cells/well) treatments. All were found to be significantly different to the +VN control (p<0.01). Interestingly, the decrease in cell number for the IGFBP-5 / IGF-I treatment between 24 hr and 48 hr was not statistically significant. This is compared to all other groups in which either a significant increase or decrease in cell number

were recorded between 24 hr and 48 hr. In addition, while there was no difference between cell numbers recorded for the IGFBP-5 / IGF-I and VN / IGFBP-5 / IGF-I treatments at 24 hr, there were significantly more cells in the VN / IGFBP-5 / IGF-I treatment compared to the IGFBP-5 / IGF-I treatment at 48 hr ($p<0.01$).

By 72 hr the 10% FCS control had increased to 9495 ± 219 cells/well, significantly more than any other treatment ($p<0.01$) (Figure 3.6c). The VN / IGFBP-5 / IGF-I treatment also had significantly more cells (8207 ± 236 cells/well) ($p<0.01$) after 72 hr compared to all other treatments except the 10% FCS control and had significantly increased in cell number between 48 hr and 72 hr ($p<0.01$). Both the VN / IGF-I and the VN / IGFBP-5 treatments resulted in significantly higher cell number, 6914 ± 225 cells/well and 6490 ± 166 cells/well respectively, than the +VN control (5382 ± 297 cells/well) ($p<0.01$). While both the VN / IGF-I and the VN / IGFBP-5 treatments significantly increased cell numbers between 48 and 72 hr ($p<0.01$) cell numbers in the +VN control did not change significantly over the same period. All wells not exposed to pre-bound VN had, as expected, significantly lower cell number compared to the +VN control at 72 hr ($p<0.01$). In addition, there were no differences in cell number between the –VN control (3466 ± 109 cells/well) and either IGF-I only (3681 ± 171 cells/well), IGFBP-5 only (3281 ± 120 cells/well) or IGFBP-5 / IGF-I (3821 ± 171 cells/well) treatments. However, the difference between the calculated cell number for the IGFBP-5 only treatment and the IGFBP-5 / IGF-I treatment was found to be statistically significant ($p<0.05$). These data indicate that while VN is able to sustain SaOS-2 survival over 72 hr, increases in cell number over time (proliferation) require the addition of either IGF-I or IGFBP-5 and may be greatly enhanced by pre-binding both IGFBP-5 and IGF-I. In addition, the absence of VN resulted in net cell loss compared to the initial cell seeding density of 5000 cells/well.

Figure 3.6. Effect of pre-bound VN, IGFBP-5 and IGF-I on SaOS-2 cell proliferation. SaOS-2 cells (5000/well) were incubated in sf-αMEM and allowed to proliferate for a) 24 hr, b) 48 hr, and c) 72 hr in wells pre-coated with solutions of IGFBP-5 (69.5 ng/well) and / or IGF-I (17.4 ng/well) in the presence or absence of pre-bound VN (52.1 ng/well). Cells seeded into wells without VN or in 10% FCS were employed as controls. Following incubation, media was aspirated and the tissue culture plates were stored at -80°C. Cyquant reagent was then added to each well as per the manufacturer's instructions and the fluorescence measured at 480 nm excitation and 520 nm emission. Results were corrected using data from plate blanks (Cyquant reagent only) and cell number calculated from a cell number standard curve and expressed as mean cell number ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significant difference to the +VN control is indicated by α ($p<0.05$) or β ($p<0.01$). Significant difference to all other groups or select groups (where linked by line) is indicated by # ($p<0.05$) or * ($p<0.01$). Horizontal red line indicates initial number of cells seeded per well.

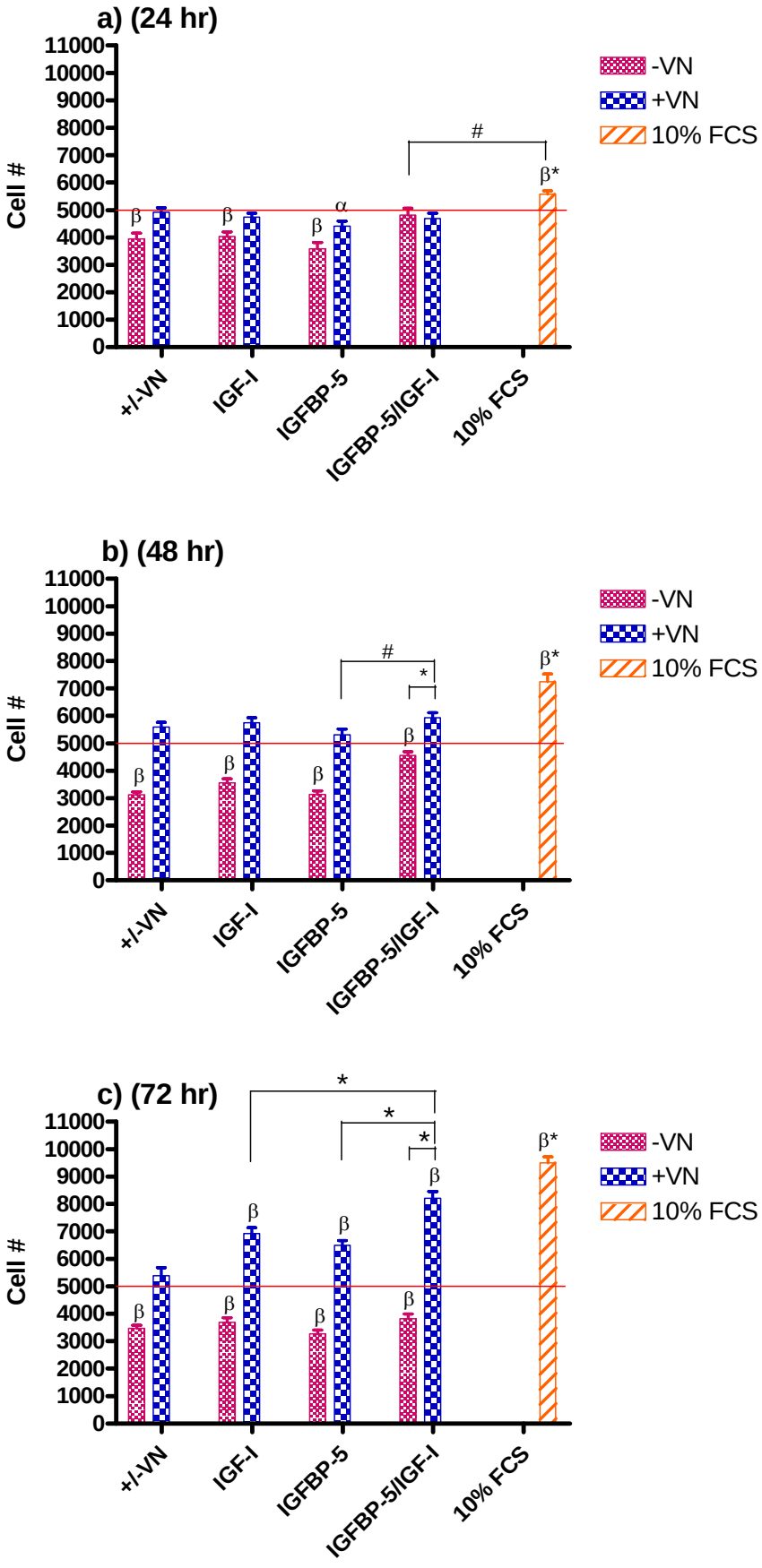

a) (24 hr)
b) (48 hr)
c) (72 hr)
Cell #
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
-VN
+VN
10% FCS
+/-VN
IGF-I
IGFBP-5
IGFBP-5/IGF-I
10% FCS
β
α
#
*
β*

Figure 3.6

3.4 DISCUSSION

In 1999 Upton *et al*. reported that IGF-II was able to bind to the ECM protein VN and that this complex retained IGF-II biological functionality. However it was also found that IGF-I was not able to bind to VN (Upton *et al.* 1999). Inspired by this initial work, Kricker *et al*. (2003) hypothesised that IGF-I may bind to VN via one or more of the 6 known IGFBPs. Significantly, this work demonstrated that IGFBP-2, -3, -4 and 5 could mediate binding of IGF-I to VN and that these complexes had functional effects on MCF-7 cell migration (Kricker *et al.* 2003). Since this work, others have reported effects on cell proliferation in HaCAT cells and primary human skin keratinocytes (Hyde *et al.* 2004; Hollier *et al.* 2005). Prior to the commencement of the present study, assessment of the ability of pre-bound combinations of VN, IGFBPs and IGF-I to elicit functional responses in cells had only been performed in epithelial type cells. Given that VN and a number of members of the IGF family of proteins, including IGF-I and IGFBP-5, are highly prevalent in bone tissue and have been demonstrated to influence osteoblast function (Bautista *et al.* 1990; Bautista *et al.* 1991; Mohan and Baylink 1991; Knutsen *et al.* 1995; Schmid *et al.* 1995; Miyakoshi *et al.* 2001; Pepene *et al.* 2001), I proposed that pre-bound combinations of VN, IGFBP-5 and IGF-I could also elicit functional responses in connective, or mesenchymal cell types.

This study has shown that when VN, IGFBP-5 and IGF-I are pre-bound to tissue culture plastic in serum-free conditions the osteoblast-like cell line SaOS-2 exhibits enhanced cell migration, metabolic activity and proliferation up to 72 hr of culture. Specifically, I have demonstrated that without pre-bound VN present, relatively few SaOS-2 cells were able to migrate through 12 μm pore Transwell™ membranes, regardless of whether IGFBP-5 or IGF-I or both were present or not (Figure 3.1). This result is similar to previous studies with MCF-7 cells and HaCAT cells reported from our laboratory (Kricker *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005). Interestingly, the HaCAT cell line migration response to complexes of IGFBP-5 and IGF-I alone has been shown to be equivalent to that of VN alone. This is likely due to the ability of HaCAT cells, to attach to tissue culture plastic without the addition of any exogenous adhesion protein such as VN (Kricker *et al.* 2003; Hyde *et al.*

2004; Hollier *et al.* 2005) whereas, as discussed below, SaOS-2 cell attachment to tissue culture plastic is dependent on the presence of an adhesion factor such as VN.

SaOS-2 cells are known to express $\alpha_v\beta_3$ integrin receptors which mediate cell attachment to a variety of ECM proteins, including VN (Koistinen *et al.* 1999; Postiglione *et al.* 2003; Kilpadi *et al.* 2004). The β_3 subunit of the $\alpha_v\beta_3$ integrin has been found to play a key role in the facilitation of the migratory response in $\alpha_v\beta_3$ integrin expressing M21 melanoma cells (Leavesley *et al.* 1992). The involvement of the β_3 subunit in facilitating cell migration was confirmed by stably transfecting a human pancreatic carcinoma cell line with the β_3 moiety, which they lacked, thus conferring the ability to migrate on a VN substrate, a function which they were previously unable to do (Leavesley *et al.* 1992).

We considered the migration response of SaOS-2 cells to VN alone (+VN) to be the basal or control response and found that addition of either IGFBP-5 or IGF-I did not change the cell migration response compared to the +VN control alone. However, SaOS-2 cells migrated strongly in response to IGFBP-5 and IGF-I together in the presence of VN. These data support findings that IGF-I is not able to bind to VN (Upton *et al.* 1999; Kricker *et al.* 2003) and that while IGFBP-5 can bind VN, it has negligible effect on cell migration in the absence of IGF-I (Kricker *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005). The significant increase in cell migration in response to VN, IGFBP-5 and IGF-I demonstrates that IGF-I, when retained on a surface by IGFBP-5 in the presence of VN, elicits strong functional effects on SaOS-2 cells. This finding may be significant because the migration response of mesenchymal type cells, such as osteoprogenitor cells or osteoblasts, is considered important for effective bone regeneration within 3 dimensional biomaterial constructs and on the surface of orthopaedic implants (Dee *et al.* 1999; Shea *et al.* 2000; Frosch *et al.* 2002; Yang *et al.* 2003a; Yang *et al.* 2003b; Schleicher *et al.* 2005).

Unlike previous studies performed in our laboratory using the HaCAT cell line, SaOS-2 cells were found to be unable to attach to tissue culture wells without the presence of VN (Figure 3.2) (Hyde *et al.* 2004; Hollier *et al.* 2005). However, this study revealed that the presence of VN facilitates SaOS-2 cell attachment to the

culture surface equivalent to that mediated by 10% FCS. Further, while the addition of IGF-I, IGFBP-5 or both, seemed to increase cell attachment, this increase was not statistically significant. These results are supported by the findings of Underwood and Bennett (1989) who demonstrated that VN is able to bind to tissue culture plastic surfaces even in the presence of other serum proteins such as FN. In addition, these authors showed that while BHK-21 cells or bovine aortic endothelial cells attached and grew normally in the presence of serum which had been stripped of FN they did not attach well to the culture surface and failed to thrive in the presence of serum which had been stripped of VN (Underwood and Bennett 1989). Thus, these data indicate that VN is the principle serum protein regulating cell attachment and growth in standard tissue culture systems.

In order to evaluate the proliferative response of SaOS-2 cells to VN, I performed a series of time course experiments using a range of doses of VN. While there was no difference in cell number between the treatments measured after 24 hr, the cells exposed to the 10% FCS treatment steadily increased in number over the following 48 hr, while cell number steadily decreased in the –VN control. Cell number did not increase nor decrease over the 72 hr in wells coated with 1 μg/mL VN or higher; indeed there was no difference in cell number between these treatments despite a four fold difference in VN concentration. However, at lower VN concentrations cell number decreased over time in a dose dependent manner. These data indicate that VN elicits a dose dependent effect on SaOS-2 cell survival over 72 hr. This result could be explained through a number of possibilities. Firstly, there may have been a steady rate of cell proliferation concomitant with a steady rate of cell death leading to no net change in cell number over 72 hr. In addition, it is possible that a significant proportion of cells had already committed to the completion of their cell cycle (Peeper *et al.* 1994; Cooper 2000). Therefore the maintenance of cell numbers over 72 hr may simply be the result of a proportion of the cells completing their cell cycle, rather than the presence of VN facilitating *bona fide* cell proliferation. Alternatively, there may have been no proliferation of cells and no cell death resulting in no net change in cell number. In which case VN will not have influenced cell proliferation, but rather, will have had a protective effect, inhibiting cell apoptosis.

VN has previously been shown to reduce microvascular endothelial cell apoptosis *in vitro* resulting in a slight increase in cell number over 72 hr. This response was a consequence of ligand occupancy of $\alpha_v\beta_3$ integrins by VN, with the α_v subunit being critical for mediation of the effect (Isik *et al.* 1998). Similar effects have been observed in osteoclasts where lack of $\alpha_v\beta_3$ ligand occupancy resulted in caspase-8 mediated apoptosis. The authors also found that caspase-8 was associated with the unoccupied $\alpha_v\beta_3$ integrin, but was not associated with this integrin in adherent cells (Zhao *et al.* 2005). More specifically, these authors and others have shown that the β_3 subunit is responsible for the recruitment and interaction with caspase-8 at the cell membrane (Stupack *et al.* 2001; Zhao *et al.* 2005). Thus a reasonable hypothesis arising from the results of the present study is that SaOS-2 cells cultured on low concentrations of pre-bound VN undergo apoptosis due to non-ligation of $\alpha_v\beta_3$ integrins and that saturating concentrations of pre-bound VN attenuate the integrin-mediated apoptotic response.

IGF-I is widely known to elicit mitogenic effects on many cell types, including osteoblasts, and the presence of IGFBP-5 is known to enhance cell responsiveness to IGF-I when bound to the culture substrate, especially when bound to ECM (Jones *et al.* 1993). Others have demonstrated similar effects in keratinocytes when IGFBP-5 together with IGF-I were pre-bound to culture substrates (Hyde *et al.* 2004; Hollier *et al.* 2005). I performed a series of assays to determine if SaOS-2 cells respond in a dose dependent manner to pre-bound VN and various concentrations of IGFBP-5 and IGF-I. After 72 hr, a clear dose dependent response to IGFBP-5 and IGF-I was evident (Figure 3.4). Indeed, the highest IGFBP-5 / IGF-I concentration facilitated an increase in cell number equivalent to 70% of that attained in the positive control, 10% FCS. In addition, IGFBP-5 / IGF-I concentrations above 115 ng/mL / 28.8 ng/mL did not significantly increase the response. A possible reason for this may be that the culture surface could not retain higher amounts of IGFBP-5 / IGF-I; that is, the culture surface / IGFBP-5 / IGF-I interaction was the limiting factor. Indeed experimental studies from our laboratory (Kricker et al. (2003), (2005)) found that that IGFBP-5 exhibited a concentration-dependent increase in binding with VN coated surfaces at concentrations <50 ng/mL and saturated at ~50 ng/mL (Kricker et al. 2003; Kricker 2005). However, the actual concentration of the

individual protein species bound to the tissue culture surface was not measured during this study.

As observed in the previous experiments the 1 µg/mL treatment (+VN control in these experiments) maintained, but did not increase, cell number above the initial cell seeding density of 5000 cells/well. VN was, however, found to be critical for facilitating the dose dependent mitogenic effect of IGFBP-5 / IGF-I. Even without VN there was a slight dose dependent response to IGFBP-5 / IGF-I, although even at the highest IGFBP-5 / IGF-I concentrations the cell number/well after 72 hr remained significantly lower than that attained for the +VN control. Thus IGFBP-5 / IGF-I complexes alone were not sufficient to maintain cell number.

To show that there had in fact been an increase in cell number for each treatment, assays were performed as a time course. Figure 3.4 inset shows the increase in cell number for the 115 ng/mL IGFBP-5 / 28.8 ng/mL IGF-I treatment, indicating a proliferative response to this treatment. All treatments exposed to VN either maintained or increased cell number by 72 hr, indicating that at least cell attachment is required for cell proliferation to occur and that this attachment factor (VN in this case) can be provided in a pre-bound form. As in the previous experiment, these data also suggest a possible role for VN in reducing SaOS-2 cell apoptosis. It is also possible that the enhanced response to the higher concentrations of IGFBP-5 / IGF-I reflects a further protective effect against apoptosis, especially if, as discussed above, the increase in cell number is partly or wholly due to a percentage of the cells having already traversed the G1 checkpoint of the cell cycle, thus committing them to S phase and DNA synthesis and not due to *bona fide* cell proliferation.

Because there is some evidence in the literature that IGFBP-5 has IGF-I independent effects on some cells, including osteoblasts, I measured the functional SaOS-2 cell response to the various combinations of VN, IGFBP-5 and IGF-I (Andress and Birnbaum 1992; Miyakoshi *et al.* 2001). Initially I decided to use WST-1, a water soluble tetrazolium salt which is cleaved by mitochondrial dehydrogenases to yield formazan, a coloured dye product which can be read spectrophotometrically and indicates cell viability. This study showed that indeed IGFBP-5 in the presence of VN could significantly, and synergistically, enhance SaOS-2 cell viability over VN

alone (Figure 3.5). Most interesting, however, was the finding that IGFBP-5 / IGF-I complexes without VN mediated an equivalent response to that of VN alone. Significantly, this indicates that IGFBP-5 / IGF-I complexes can bind to the culture surface without VN and subsequently influence cellular metabolic activity to a much greater extent than either of the 2 components in isolation. Others have also recorded elevated functional responses to IGFBP-5 / IGF-I complexes alone, indicating that perhaps IGFBP-5 / IGF-I complexes bind to the tissue culture surface and that this complex retains IGF-I bioactivity (Hyde *et al.* 2004; Hollier *et al.* 2005). Further, IGFBP-5 has been shown to mediate binding of [^{125}I]-IGF-I to VN in solid plate binding assays. However, IGFBP-5 was not able retain [^{125}I]-IGF-I tracer in the absence of VN (after the wells were blocked with 0.5% BSA) indicating that IGFBP-5 could not bind to the well surface (Kricker *et al.* 2003). These solid plate binding studies were performed on high protein binding plastic rather than tissue culture plastic thus the efficacy of BSA in blocking the non-specific binding of IGFBP-5 may account for the differences. Because in the present study 'tissue culture' modified polystyrene plates were used, it is possible that some IGFBP-5 / IGF-I complexes bind to VN, while others bind to the tissue culture surface. Indeed IGFBP-5 is known to be a very "sticky protein" and it is likely that this property explains the apparent retention of biologically active protein at the culture surface in this and previous studies (Gropep; Hyde *et al.* 2004; Hollier *et al.* 2005). As described above, solid plate binding studies previously performed in our laboratory have shown that IGFBP-5 binds to VN in a concentration dependent manner, saturating at higher doses (Kricker *et al.* 2003; Kricker 2005). However, it is not known how much IGFBP-5 / IGF-I binds to tissue culture plastic, nor what concentrations of IGFBP-5 and IGF-I are optimal for the binding interaction in our system. As far as I am aware this has not been investigated previously.

Investigation of the effect of the various combinations of VN, IGFBP-5 and IGF-I on cell proliferation revealed that all treatments without VN resulted in a steady decrease in cell number over time. In contrast, treatments which incorporated VN resulted in either an increase in cell number (VN / IGFBP-5 / IGF-I, VN / IGFBP-5 and VN / IGF-I) or maintenance in cell number (+VN control) as described above for previous experiments (Figure 3.6 a, b and c). Curiously, the trends revealed in Figure 3.5, which were measured after 48 hr culture, more closely mirror the trends

for the cell number data collected at 72 hr of culture (Figure 3.6c). Together these data suggest that at 48 hr SaOS-2 cells exposed to VN, IGFBP-5 and IGF-I were actively proliferating, whereas the other VN treatments resulted in at most, only slightly increased cell number. Thus the metabolic activity of the cells at 48 hr were reflected in terms of cell number 24 hr later ie. at 72 hr. This has implications for the common use of reagents such as tetrazolium salts as indicators of cell number since the present study shows there is a clear discrepancy between the WST-1 results and cell number results at 48 hr.

Interestingly, IGF-I ligation of the IGF-IR has been shown to simultaneously promote the mitogenic pathway and anti-apoptotic signalling pathways via parallel PI-3 kinase and p42 / 44 MAPK signalling in SaOS-2 cells (Grey *et al.* 2003). In addition, IGFBP-5 and IGF-I together have been shown to significantly enhance the mitogenic response of various connective tissue cells, particularly when the IGFBP-5 / IGF-I complex is immobilised on the culture surface via the ECM (Andress and Birnbaum 1992; Jones *et al.* 1993). This is believed to occur because IGF-I has a reduced affinity for IGFBP-5 when IGFBP-5 is bound to the ECM, compared to IGFBP-5 in solution, therefore cells are better able to access or sequester the IGF-I from the bound IGFBP-5. Localisation of the complex in the vicinity of the cell layer, along with the reduced affinity of IGF-I for bound IGFBP-5, would drive the IGF equilibrium away from the binding protein and toward the receptor, thus resulting in enhanced interaction between IGF-I and its receptor and the observed enhanced responses of cells cultured on bound IGFBP-5 / IGF-I complexes (Jones *et al.* 1993).

Moreover, a number of studies have investigated the interaction between the IGF1R signalling pathway and the $\alpha_v\beta_3$ integrin signalling pathway. Exposure of smooth muscle cells to VN has been shown to increase the degree of tyrosine phosphorylation of IRS-1, a member of the IGF1R signalling pathway, in response to IGF-I stimulation (Zheng and Clemmons 1998). The same effect was not seen when cells were plated on laminin / type IV collagen matrix, which bind $\alpha2\beta1$ integrins, indicating that the effect of VN on the IGF1R is specifically mediated by $\alpha_v\beta_3$ integrins. This was further supported by blocking $\alpha_v\beta_3$ with echistatin which

significantly reduced IRS-1 and 2 tyrosine phosphorylation and association of the p85 subunit of PI-3 kinase with IRS-1 (Zheng and Clemmons 1998). More recently, various components of the β_3 subunit of the $\alpha_v\beta_3$ integrin, in particular its cytoplasmic tail, has been identified as being required for the modulation of IGF-I mediated phosphorylation of the IGF1R, further supporting the interrelationship of $\alpha_v\beta_3$ integrin and IGF1R signalling pathways (Maile *et al.* 2001). Therefore I hypothesise that a similar mechanism of cross talk exists in osteoblasts, including SaOS-2 cells, thus providing a mechanistic explanation for the results detailed in this chapter. Furthermore, it is likely that the increase in cell migration, metabolic activity and cell number in response to VN, IGFBP-5 and IGF-I is a combination of protection from apoptosis by VN via ligation to $\alpha_v\beta_3$ or other integrins, IGF-I mediated anti-apoptotic signalling and the mitogenic action of IGF-I.

In conclusion, the initial responses of SaOS-2 cells to pre-bound VN, IGFBP-5 and IGF-I detailed in this chapter indicate that these proteins together hold potential as a ECM / growth factor coating for biomaterial scaffolds in order to induce cell responses such as cell migration into and proliferation within the scaffold.

CHAPTER 4:

EFFECTS OF SOLUTION PHASE VITRONECTIN, IGFBP-3 OR -5 AND IGF-I OR TGF-β_1 ON hMSC METABOLIC ACTIVITY, MORPHOLOGY AND GELATINASE EXPRESSION.

4.1 INTRODUCTION

Over the last 2 decades human mesenchymal stem cells have become integral constituents of many modern tissue engineered constructs for bone or connective tissue repair (Otto and Rao 2004). This is due to their demonstrated ability to facilitate regeneration of mesenchymal tissues including bone, cartilage, adipose tissue, muscle, marrow stroma and their capacity for self renewal (Bruder *et al.* 1997; Jaiswal *et al.* 1997; Pittenger *et al.* 1999; Lennon *et al.* 2000). Differentiation of hMSCs into these various tissue types is largely controlled by a complex interplay of specific growth factors, cytokines, vitamins and other factors in the microenvironment to which they are exposed, such as ECM molecules (Bruder *et al.* 1997; Jaiswal *et al.* 1997; Pittenger *et al.* 1999; Coelho *et al.* 2000; Coelho and Fernandes 2000; Lennon *et al.* 2000; Bennett *et al.* 2001). Some modern strategies for the development of connective tissue therapeutics focus on implantable constructs which consist of some form of ECM bound to a biomaterial scaffold, often with *ex vivo* expanded, undifferentiated hMSCs. These constructs are designed to deliver large numbers of precursor cells to the site of injury, which will differentiate into appropriate tissue types through interactions with the surrounding host tissue and the accompanying scaffold / ECM (Bruder *et al.* 1998b; Bruder *et al.* 1998c; Young *et al.* 1998; Ponticiello *et al.* 2000). MSCs that have been expanded *in vitro* and seeded onto biodegradable collagen scaffolds and implanted into large segmental bone defects in mice have been shown to synthesise new bone (Turgeman *et al.* 2001). Similarly, MSCs seeded onto β-tricalcium phosphate scaffolds which were then implanted into significant femoral defects in rats, support bone regeneration with superior properties than the scaffold alone (Bruder *et al.* 1998c). Thus, tissue engineered constructs which contain incorporated MSCs have the ability to regenerate bone tissue in critical sized defects in animal models, this in turn strongly suggests this approach has an equivalent potential in humans. These data support my own view, that hMSCs which are expanded *ex vivo* and seeded into tissue engineered constructs which have been coated with appropriate matrix / growth factors will enhance integration of the implant into the repair site. Therefore, characterisation of the responses of *ex vivo* expanded hMSC interacting with surface bound ECM in the presence of some of the most highly expressed growth factors in bone tissue is of great interest.

The major matrix component of bone is type I collagen, although adult bone is also rich in other matrix proteins such as fibronectin and to a lesser extent VN (Grzesik and Robey 1994; Seiffert 1996). In addition, the IGF–I and –II along with the IGF binding protein, IGFBP-5, and TGF-β_1 are among the most abundent growth factors in bone (Bautista *et al.* 1991; Andress and Birnbaum 1992; Hakeda *et al.* 1996; Franchimont *et al.* 1997a; Franchimont *et al.* 1997b). Recently, the IGFs together with the IGFBPs have been shown to interact with each other and the matrix protein VN *in vitro* and to stimulate functional responses in a number of epithelial cell types (Kricker *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005). TGF-β_1 has also recently been shown to associate with VN and retain functional effects on cells (Schoppet *et al.* 2002). Furthermore, interactions between the ECM and components of the IGF system and other growth factors such as TGF-β_1 are believed to affect hMSC function and bone tissue growth and turnover (Golombick *et al.* 1995; Martin *et al.* 2002; Longobardi *et al.* 2003; Govoni *et al.* 2005; Minuto *et al.* 2005; Kiepe *et al.* 2006), thus facilitating interest in the potential use of these compounds in the development of novel tissue engineered therapeutics. Importantly, hMSCs are known to express a range of integrins which include $\alpha_v\beta_3$ integrins (the VN receptor) and also express growth factor receptors including the IGF-IR, and receptors for TGF-β_1 (Conget and Minguell 1999). Some studies have suggested that VN can induce osteogenic differentiation in hMSCs (Salasznyk *et al.* 2004a; Salasznyk *et al.* 2004b), while TGF-β_1 has been shown to mediate proliferative responses in hMSCs through induction of IGFBP-3 and IGF-I expression which act in an autocrine manner (Kveiborg *et al.* 2001b). TGF-β_1 has also been implicated in the mediation of differentiation responses in transitory stage osteoblasts but seems to inhibit the expression of some osteogenic markers such as osteocalcin in later secretory stage osteoblasts (Kassem *et al.* 2000). Thus the effects of TGF-β_1 on hMSC and osteoblast function are complex and obscure.

There is little data available on the effects of IGFBP-5 on hMSCs despite the finding that hMSCs express both IGFBP-3 and -5 and IGF-I (Kveiborg *et al.* 2001b) and that both IGFBP-5 and IGF-I are down regulated by the pro-differentiation steroid dexamethasone (Cheng *et al.* 1998). However, IGFBP-5 and its proteolytic fragments have been shown to elicit IGF independent effects on osteoblast cell

function in mouse and human cells, including proliferation and the phosphorylation of serine residues on a 420 kDa membrane bound putative IGFBP-5 receptor (Andress 1995a; b; 1998).

Another major impediment for the re-implantation of culture expanded hMSCs for therapeutic use *in vivo* is that current culture methodologies require the use of animal sera to supply appropriate nutrients and growth factor / ECM components required to support cell viability and growth. This poses a number of risks including the xenobiotic transfer of disease, and accounts for the increasing regulatory constraints on using cells which have been cultured in animal sera for therapeutic applications (Federal Register). As such a number of studies have applied various strategies as an attempt to address this issue, including the use of patient-derived sera (Stute *et al.* 2004; Cenni *et al.* 2005) or platelet rich plasma (a rich source of growth factors) (Weibrich *et al.* 2002; Sanchez *et al.* 2003; Yazawa *et al.* 2003). However, regulatory bodies find such undefined media components also undesirable. Thus there is an urgent need for the development of highly defined culture media for *ex vivo* expansion of hMSCs without the use of animal products. In light of this and the natural abundance of members of the IGF system, TGF-β_1 and the presence of VN in bone, I believe that the combination of these proteins may have potential for replacing serum for *ex vivo* expansion of hMSCs.

4.2 EXPERIMENTAL PROCEDURES

Full details of both the materials and methods used in the generation of data presented in this chapter have been described in chapter 2. The following are brief summaries of the materials and experimental procedures used for the generation of data presented in section 4.3.

4.2.1 Materials

Purified human VN, IGFBP-5, IGF-I, WST-1 reagent, 96-well tissue culture plates and Fraction V RIA grade BSA were obtained as detailed in the previous chapter and the Materials and Methods chapter. MMP-2 and MMP-9 standards were purchased from Chemicon Pty / Ltd (Boronia, Vic, Australia).

4.2.2 Isolation and culture of hMSC from donor bone marrow samples.

For full details of this method please refer to section 2.2.2. Bone marrow samples were collected, following informed consent and with hospital and university ethics committee approvals, from both female and male patients presenting for total knee or hip joint replacement surgery as part of their treatment for osteoarthritis at The Prince Charles Hospital and The Holy Spirit Northside Hospital, Brisbane. Typically 2-5 mL of bone marrow was collected during surgery by the surgeon into tubes containing 5 mL PBS supplemented with 200 U/mL heparin and the tubes were placed on ice for transport to QUT. Collagenase B (0.02%) and DNase (100 U/mL) were occasionally employed to assist in disaggregation of clots, otherwise the marrow suspension was repeatedly passed through a 19 g needle to dislodge clumped cells. The samples were then diluted to 30 mL with PBS, thoroughly mixed and passed through a 100 µm filter to remove extracellular debris and bone fragments. Following overlaying of 30 mL of the filtrate onto 15 mL Lymphoprep™, the samples were centrifuged at 400 g for 35 min at 20°C with zero brake. The mononuclear cell fraction (buffy coat) was removed, placed into a fresh tube with 20 mL $DMEM^+$ and centrifuged at 1000 rpm for 10 min. The cell pellet was resuspended in 1 mL of $DMEM^+$ (for culture) or sf-DMEM (for CFU-F assays) prior to counting in a haemocytometer. Cells were then seeded into tissue culture flasks at 1.6×10^5 cells/cm^2 and incubated in a humidified 5% CO_2/95% air atmosphere at 37 °C. Culture media was changed after 5 days in the first instance, then twice weekly.

4.2.3 Colony Forming Unit-Fibroblastic Assay

For full details of this method please refer to section 2.10. While the growth factor concentrations were maintained as described for the last chapter, the concentration of VN was increased to see if the addition of a higher concentration of VN would affect the response of hMSCs to any of the treatments. Specifically, 1.4 mL of 4 µg/mL (5.6 µg/well) VN / sf-DMEM solution was added to each well of a 6-well tissue culture plate and incubated in a humidified 5% CO_2/95% air atmosphere at 37 °C. Following incubation for 2.5 hr, the VN solution was removed and 1.4 mL of 0.16 µg/mL (224 ng/well) TGF-β_1, or 1.6 µg/mL (2.24 µg/well) IGFBP-3 or -5 in 0.5%BSA / HBB, alone or in combination with 0.4 µg/mL (560 ng/well) IGF-I, were added to wells with VN and incubated at 4 °C overnight. Growth factor solutions

were removed prior to seeding with freshly harvested bone marrow mononuclear cells at a density of 1.6 x 10^5 cells/cm^2 in a final volume of 3 mL/well or 3.4 mL/well for solution phase studies. For the solution phase studies, VN was allowed to pre-bind as described above, however, the growth factors were left *in situ* upon seeding of cells. Therefore the final growth factor concentrations for the solution phase studies were 0.066 µg/mL (2.6 nM) (TGF-β_1) or 0.659 µg/mL (22 nM) (IGFBP-3 or -5) either alone or in combination with 0.164 µg/mL (22 nM) (IGF-I).

4.2.4 Preparation of tissue culture plates with VN and growth factors for metabolic activity and total protein assays.

For full details of this method please refer to section 2.5. hMSC cell metabolic activity and total protein were assessed in the presence of solution phase combinations of VN, IGFBP-3 or -5 and IGF-I or TGF-β_1. Specifically, 52 µL of 1 µg/mL (52 ng/well) or 3.346 µg/mL (174 ng/well) VN / sf-DMEM solution or sf-DMEM alone was added to each well of 96 well tissue culture plates and incubated for 2 hrs in a humidified 5% CO_2/95% air atmosphere at 37°C. Following incubation, 43.5 µL of 0.16 µg/mL (7 ng/well) TGF-β_1 or 1.6 µg/mL (70 ng/well) IGFBP-3 or -5 in 0.5%BSA / sf-DMEM, either alone or in combination with 0.4 µg/mL (17.4 ng/well) IGF-I, were added to wells with or without VN and incubated at 4°C overnight. Plates were then allowed to return to room temperature prior to seeding of cells. Final concentrations of added proteins following seeding of cells (final volume of 195.5 µL) were 0.266 µg/mL (3.55 nM) or 0.890 µg/mL (11.9 nM) VN, 0.358 µg/mL (11.9 nM) IGFBPs, 89 ng/mL (11.9 nM) IGF-I and 36 ng/mL (1.43 nM) TGF-β_1.

4.2.5 Metabolic Activity Assay

For full details of this method please refer to section 2.9. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding of 5000 cells/well into 96 well plates containing various combinations of VN, IGFBP-5 and IGF-I in a final volume of 195.5 µL. Cells were allowed to incubate for 24 hr, 48 hr or 72 hr prior to removal of conditioned media and addition of 110 µL of WST-1 reagent / SF-DMEM solution to each well. Absorbance readings at 450 nm - 650 nm were taken following a 2 hr incubation. Results are from 4 separate experiments with each

treatment performed in at least triplicate and are expressed as the corrected absorbance at 450 nm - 650 nm as a percentage of the 24 hr -VN control ± standard error of the means (SEM).

4.2.6 Total Protein assay

For full details of this method please refer to section 2.11. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding of 5000 cells/well into 96 well plates containing various combinations of VN, IGFBP-5 and IGF-I in a final volume of 195.5 µL. Cells were allowed to incubate for 24 hr, 48 hr or 72 hr. Conditioned media was then removed and cell monolayers were fixed with 4% paraformaldehyde solution, incubated for 20 min and subsequently stained with crystal violet. The plates were immersed in a beaker of gently circulating tap water and destained until no further stain visibly eluted from the wells. The plates were then allowed to air dry o/n after which the crystal violet stain was solubilised with 10% acetic acid and absorbance readings were taken at 595 nm. Results are from 4 separate experiments with each treatment performed in at least triplicate and are expressed as the corrected absorbance at 595 nm as a percentage of the 24 hr -VN control ± standard error of the means (SEM).

4.2.7 Morphological analysis

For full details of this method please refer to section 2.12. Photographic images depicting culture morphology were captured from air dried cultures prepared for the total protein assay (above), just prior to solubilisation of crystal violet with 10% acetic acid. A single image of each well was taken at X100 magnification with a Nikon Cool pix digital camera mounted on a Nikon TE100 reverse stage, phase contrast microscope.

4.2.8 Gelatin Zymography

For full details of this method please refer to section 2.13. Conditioned media was removed from representative wells of the total protein assays immediately prior to fixation of cells with paraformaldehyde and stored at -20 °C until required for analysis. Samples were then analysed for MMP-2 and MMP-9 gelatinolytic activity. Briefly, conditioned media samples were applied to 10% polyacrylamide gels containing 1 mg/mL gelatin and subjected to electrophoresis at 4 °C. The gels were

washed in 2.5% Triton-X100 to remove SDS and then incubated in incubation buffer (50 mM Tris base, 10 mM $CaCl_2$, 50 mM NaCl, pH 7.6) for 48 hr at 37 °C. Gelatinase activity was visualised by staining the gels with Coomasie Blue R250 and destained in 10% acetic acid and 40% methanol (vol/vol).

4.2.9 Western blot of IGFBP-5 degradation by hMSC conditioned media

For full details of this method please refer to section 2.14. The conditioned media from hMSCs cultured in sf-DMEM alone was collected and stored as above. Samples of serum free hMSC conditioned media, phosphate buffered saline or sf-DMEM were incubated with or without 400ng of recombinant human IGFBP-5 o/n at 37° C and then separated under reducing conditions on a 4%-20% gradient polyacrylamide gel. Proteins were then transferred to a nitrocellulose membrane prior to interrogation with a chicken anti IGFBP-5 polyclonal antibody and detection with a rabbit anti chicken-HRP conjugated 2° antibody and chemiluminescent substrate. Chemiluminescence was then recorded onto photographic film.

4.3 RESULTS

4.3.1 Effect of pre-bound or solution phase VN, IGFBPs and IGF-I on bone marrow derived fibroblastic cell attachment.

The colony forming unit fibroblastic assay (CFU-F) has been used for many years as a means of determining the efficiency of hMSC colony formation from the mononuclear cell fraction of whole bone marrow (Owen and Friedenstein 1988; Gronthos and Simmons 1995; Majumdar *et al.* 2000; Meuleman *et al.* 2006). In order to determine if pre-bound combinations of VN and either IGFBP-3 or -5 and IGF-I or TGF-β_1 could initiate bone marrow derived fibroblast colony formation, I performed an initial colony forming unit fibroblast assay. In this experiment I observed that after 7 days almost no cells were attached to the culture surface of any wells containing any combination of pre-bound protein; thus figure 4.1a & b show the VN / IGFBP-3 / IGF-I treatment at 1 and 7 days and is typical of the response obtained for the other pre-bound treatments (Fig 4.1a & b). In contrast, cells cultured in the presence of 10% FCS had established a number of distinct colonies by day 1, which had then expanded in size by day 7 with clearly visible fibroblast-like cells in the vicinity of the colony (Fig 4.1c & d). These cultures continued to thrive and

further develop into near confluent cultures by day 13 (data not shown). This indicates that the cells used for these studies were viable under standard culture conditions and that pre-bound treatments of VN and / or IGFBPs and / or IGF-I and / or TGF-β_1 failed to support the adhesion of fibroblastic cells from bone marrow. Based on these observations I investigated whether pre-bound VN with solution phase IGFBP-3 or -5 and / or IGF-I or TGF-β_1 could support CFU-F formation from bone marrow. This approach indeed, resulted in attachment of many elongated shaped cells after the first day, which, then went on to form spindle shaped fibroblastic cells by day 3 (Fig 4.1e-h). Interestingly, the fibroblast like cells in these cultures did not form, nor seem to arise from colonies. In fact none of the treatments induced colony formation except the 10% FCS control which, by day 13, as stated above, had induced large numbers of fibroblastic cells in large colonies (data not shown). In addition, only the 10% FCS culture continued to thrive after 3-4 days of culture. In contrast, cells cultured in the presence of VN and solution phase growth factors declined in number from about day 4 and few viable cells remained after day 7 (data not shown).

Figure 4.1. Effect of pre-bound or solution phase VN, IGFBPs and IGF-I on bone marrow derived fibroblastic cell attachment. Whole bone marrow from patients undergoing orthopaedic surgery was obtained with informed consent. The mononuclear cell fraction was seeded onto pre-bound VN, IGFBP-3 and IGF-I (a & b) or pre-bound VN with solution phase IGFBP-3 (e & f) or IGFBP-5 (g & h) and IGF-I at a density of 1 x 10^6cells/cm^2 and incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for up to 13 days. Mononuclear cells cultured in the presence of 10% FCS served as positive control cultures (c & d). Photographs of cultures were taken at X200 magnification on day 1 and then as indicated. Bar = 100µm.

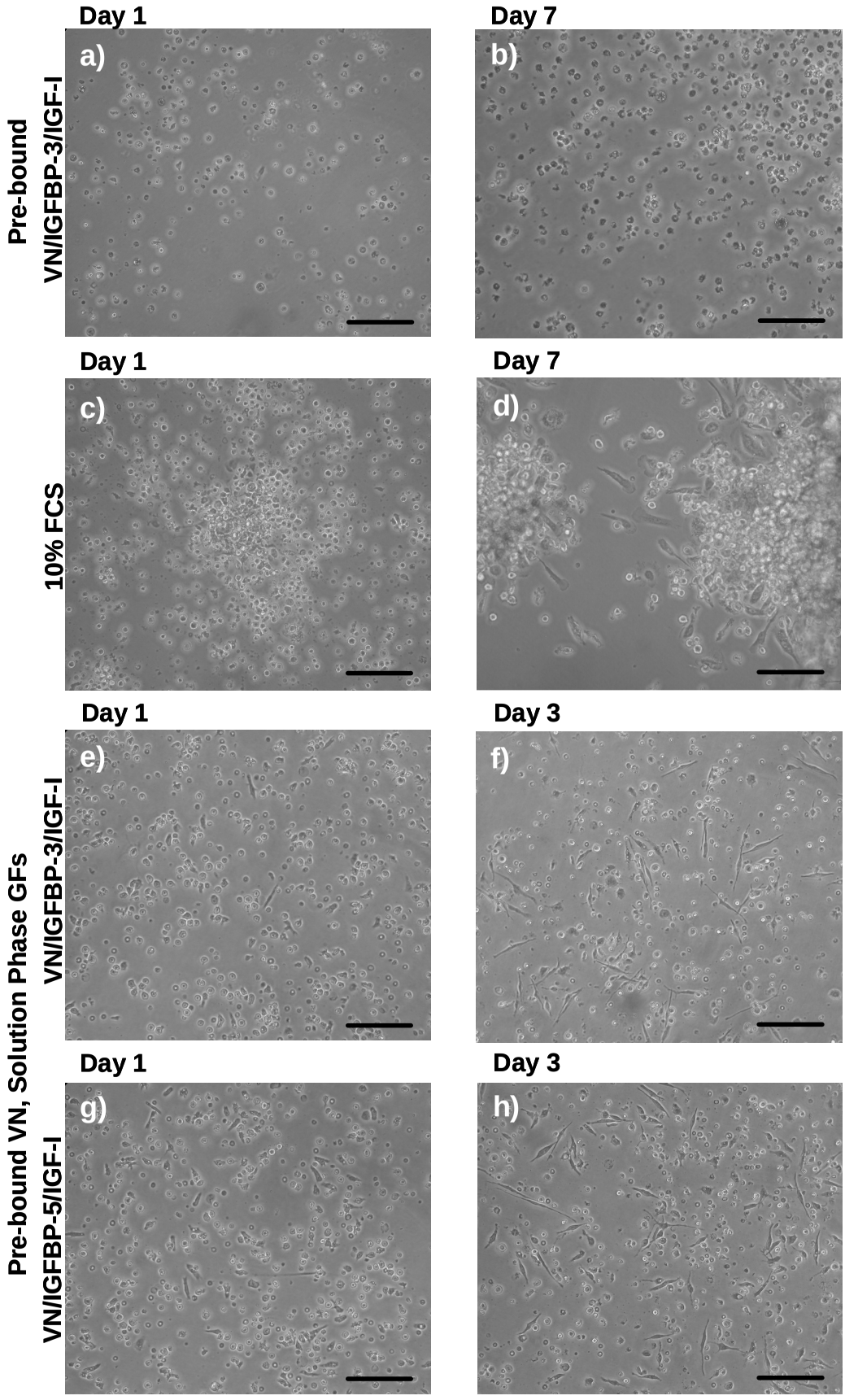

Day 1
Day 7
Pre-bound VN/IGFBP-3/IGF-I
a)
b)
Day 1
Day 7
10% FCS
c)
d)
Day 1
Day 3
Pre-bound VN, Solution Phase GFs VN/IGFBP-3/IGF-I
e)
f)
Day 1
Day 3
Pre-bound VN/IGFBP-5/IGF-I
g)
h)

Figure 4.1

4.3.2 Effect of solution phase VN, IGFBP-5 and IGF-I on hMSC metabolic activity.

Because the mononuclear fibroblastic cells had failed to thrive after 4 days of culture I elected to use hMSCs, which had been established in culture, for subsequent experiments. In addition, I decided to pre-bind VN to the culture surface as I had done previously but to leave the un-bound fraction of VN and growth factors in solution. Furthermore, as stated above I also wanted to examine if the addition of a higher concentration of VN would affect the response of hMSCs to any of the treatments.

In the first instance, I found that hMSCs from established cultures, attached and spread well on all treatments in which VN was present. In contrast, where VN was absent the cells were rounded and appeared to be poorly attached. I measured the metabolic activity of hMSCs at 24 hr, 48 hr and 72 hr and found that after 72 hr there was no difference in the response of hMSCs to VN alone at either 52 ng/well (3.55 nM), or 174 ng/well (11.9 nM) (Fig 4.2a). Indeed the VN concentration did not affect the metabolic activity response of hMSCs to any of the treatments unless it was absent altogether. However, VN alone at 52 ng/well, or at 174 ng/well (108.3 ± 15.0% (n=11) or 105.8 ± 17.3% (n=12) of 24 hr –VN control respectively) resulted in significantly higher metabolic activity after 72 hr than hMSCs exposed to sf-DMEM alone (72 hr –VN control) (64.5 ± 8.5% (n=16) of 24 hr –VN control)(p<0.05). Similarly, IGF-I in the presence of VN at 52 ng/well, or 174 ng/well (130.7 ± 18.4% (n=11) or 121.9 ± 16.4% (n=12) of 24 hr –VN control respectively), resulted in significantly higher metabolic activity after 72 hr than the 72 hr –VN control (p<0.01), as did hMSCs exposed to IGFBP-5 / IGF-I in the presence of VN at 52 ng/well, or 174 ng/well (104.4 ± 18.6% (n=11) or 110.6 ± 16.0% (n=12) of 24 hr –VN control respectively)(p<0.01). In contrast, cultures exposed to IGFBP-5 alone but in the presence of VN at 52 ng/well, or 174 ng/well (92.0 ± 15.9% (n=11) or 96.7 ± 15.0% (n=12) of the 24 hr –VN control respectively), were not significantly different to the 72 hr –VN control. Interestingly, of the treatments containing VN, only the VN / IGFBP-5 treatments were statistically different to the response obtained for hMSCs exposed to 10% FCS (133.0 ± 8.0% (n=24) of the 24 hr –VN control)(p<0.05) (Fig 4.2a). These data

indicate that after 72 hr, VN was the major factor contributing to hMSC metabolic activity.

However, analysis of the 174 ng/well VN time course data revealed that after 24 hr, the metabolic activity of hMSCs exposed to VN alone (150.3 ± 10.3% (n=12)), VN / IGFBP-5 / IGF-I (157.8 ± 10.3% (n=12)) or 10% FCS (155.0 ± 13.9% (n=30)) was significantly greater than the 24 hr –VN control (100.0 ± 1.4 % (n=17))($p<0.01$). Surprisingly, the IGFBP-5 / IGF-I treatment (71.5 ± 2.8% (n=17)) sustained less metabolic activity than the 24 hr –VN control ($p<0.01$)(Fig 4.2b and Table 4.2.1). All treatments resulted in a decrease in metabolic activity over the next 48 hr prior to stabilising by 72 hr. Only in the presence of 10% FCS was a significant recovery of the metabolic activity observed between 48 hr and 72 hr (105.2 ± 3.0% (n=27) and 133.0 ± 8.0% (n=24) of the 24 hr –VN control respectively)($p<0.01$). However, the metabolic activity of the 10% FCS control measured after 72 hr was not significantly different to the activity after 24 hr, indicating that the hMSCs had recovered from the decrease observed between 24 hr and 48 hr.

Figure 4.2. Effect of solution phase VN, IGFBP-5 and IGF-I on hMSC metabolic activity. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM or 11.9 nM VN (52 ng/well or 174 ng/well), 11.9 nM IGFBP-5 (70 ng/well) and 11.9 nM IGF-I (17.4 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr b), 48 hr b) or 72 hr a) & b) in a humidified 5% CO_2/95% air atmosphere at 37°C prior to removal of conditioned media, addition of 110 µL of WST-1 / sf-DMEM solution and incubation in the same conditions for a further 2 hr. The absorbance of the coloured formazan cleavage product was quantitated at 450 nm using 650 nm as reference. Results were corrected using data from plate blanks (serum free media and WST-1 alone) and are expressed as the mean corrected absorbance as a percentage of the 24 hr –VN control (depicted by horizontal red line) ± standard error of the means (SEM). Results are from 4 separate experiments. a) Depicts results obtained at 72 hr, while significance (post-hoc t-test) between individual treatments and the 72 hr –VN control or between individual treatments where connected by line is indicated by α (p<0.05) or β (p<0.01). b) Depicts time course results obtained for –VN, VN at 174ng/well (+VN) and ±VN / IGFBP-5 / IGF-I (±VN / 5 / I) treatments while significance between treatments is indicated by * (p<0.05) or # (p<0.01) and are presented below in table 4.2.1.

Table 4.2.1 Summary of statistical analysis for Figure 4.2b

hMSC metabolic activity response to VN/BP-5/I (Time course)		% of -VN @ 24 hr ± SEM	24 hr					48 hr					72 hr				
			-VN	+VN	BP-5/I	VN/BP-5/I	10% FCS	-VN	+VN	BP-5/I	VN/BP-5/I	10% FCS	-VN	+VN	BP-5/I	VN/BP-5/I	10% FCS
24 hr	-VN	100 ± 1.4															
	+VN	150.3 ± 10.3	#														
	BP-5/I	71.5 ± 2.8	#	#													
	VN/BP-5/I	157.8 ± 10.3	#	ns	#												
	10% FCS	155.0 ± 13.9	#	ns	#	ns											
48 hr	-VN	58.5 ± 9.4	#	#	ns	#	#										
	+VN	93.4 ± 12.0	ns	#	*	#	*	*									
	BP-5/I	43.2 ± 8.8	#	#	#	#	#	ns	#								
	VN/BP-5/I	105.9 ± 11.4	ns	#	#	#	*	#	ns	#							
	10% FCS	105.2 ± 3.0	ns	#	#	#	#	#	ns	#	ns						
72 hr	-VN	64.5 ± 8.5	#	#	ns	#	#	ns	ns	ns	#	#					
	+VN	105.8 ± 17.3	ns	*	*	*	ns	*	ns	#	ns	ns	*				
	BP-5/I	48.9 ± 9.2	#	#	*	#	#	ns	#	ns	#	#	ns	#			
	VN/BP-5/I	110.6 ± 16.0	ns	*	#	*	ns	#	ns	#	ns	ns	*	ns	#		
	10% FCS	133.0 ± 8.0	#	ns	#	ns	ns	#	#	#	ns	#	#	ns	#	ns	

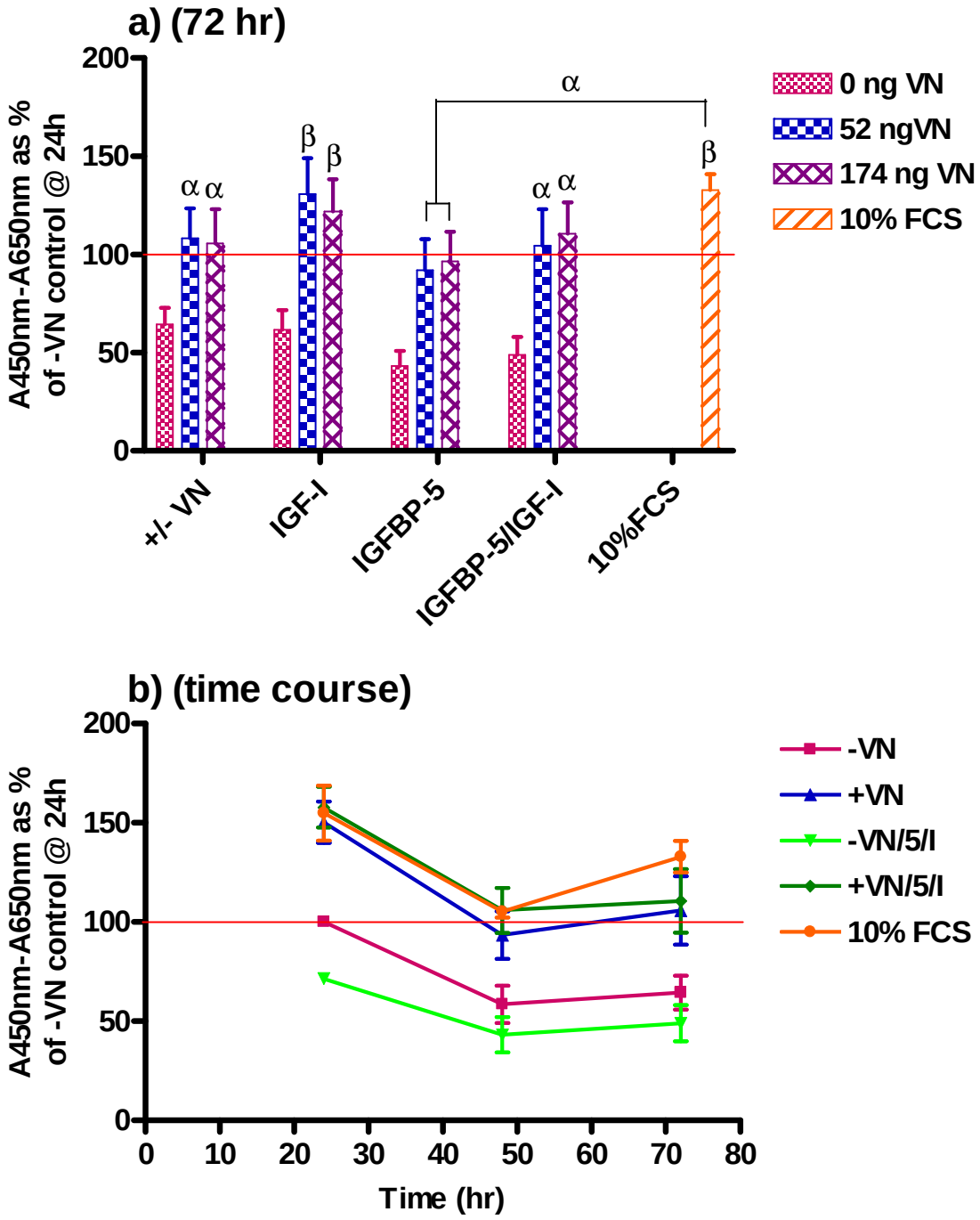

a) (72 hr)
A450nm-A650nm as % of -VN control @ 24h
200
150
100
50
0
α α
β β
α α
α
β
0 ng VN
52 ngVN
174 ng VN
10% FCS
+/- VN
IGF-I
IGFBP-5
IGFBP-5/IGF-I
10%FCS
b) (time course)
A450nm-A650nm as % of -VN control @ 24h
200
150
100
50
0
0 10 20 30 40 50 60 70 80
Time (hr)
-VN
+VN
-VN/5/I
+VN/5/I
10% FCS

Figure 4.2

4.3.3 Effect of solution phase VN, IGFBP-3 and IGF-I on hMSC metabolic activity.

hMSCs exposed to IGFBP-3 / IGF-I in the presence of VN at 52 ng/well or 174 ng/well (114.0 ± 18.0% (n=11) or 119.3 ± 16.7% (n=12) of 24 hr –VN control respectively) were also examined and cells were found to exhibit significantly higher metabolic activity than the 72 hr –VN control (64.5 ± 8.5% (n=16) of the 24 hr –VN control) ($p<0.01$), indicating that IGFBP-3 / IGF-I has a similar effect on hMSC metabolic activity to IGFBP-5/IGF-I. However, unlike IGFBP-5, cultures exposed to IGFBP-3 alone but in the presence of VN at 52 ng/well, or 174 ng/well (99.2 ± 15.6% (n=11) or 97.7 ± 14.8% (n=12) of the 24 hr –VN control respectively) possessed a significantly higher metabolic activity after 72 hr compared to the 72 hr –VN control ($p<0.05$) (Fig 4.3a). In addition, the VN / IGFBP-3 treatments yielded a response that was statistically different to the response observed when hMSCs were exposed to 10% FCS (133.0 ± 8.0% (n=24) of the 24 hr –VN control)($p<0.05$). Thus after 72 hr, VN was the major factor contributing to measurable hMSC metabolic activity and the addition of either IGFBP-5, or IGFBP-3, with IGF-I could not stimulate metabolic activity over that obtained by VN alone or VN together with IGF-I (Fig 4.3a).

Once again, analysis of the 174 ng/well VN data revealed that after 24 hr the metabolic activity of hMSCs exposed to VN / IGFBP-3 / IGF-I (165.1 ± 9.9% (n=12)) was significantly higher than the 24 hr –VN control (100.0 ± 1.4 % (n=17)($p<0.01$), while cells treated with IGFBP-3 / IGF-I (80.5 ± 2.7% (n=17)) exhibited significantly lower metabolic activity ($p<0.01$) (Fig 4.3b and Table 4.3.1). As previously observed and noted the metabolic activity of hMSCs exposed to any treatment containing IGFBP-3 decreased between 24 hr and 48 hr and then stabilised by 72 hr.

Figure 4.3. Effect of solution phase VN, IGFBP-3 and IGF-I on hMSC metabolic activity. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM or 11.9 nM VN (52 ng/well or 174 ng/well), 11.9 nM IGFBP-3 (70 ng/well) and 11.9 nM IGF-I (17.4 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr b), 48 hr b) or 72 hr a) & b) in a humidified 5% CO_2/95% air atmosphere at 37°C prior to removal of conditioned media, addition of 110 µL of WST-1 / sf-DMEM solution and incubation in the same conditions for a further 2 hr. The absorbance of the coloured formazan cleavage product was quantitated at 450 nm using 650 nm as reference. Results were corrected using data from plate blanks (serum free media and WST-1 alone) and are expressed as the mean corrected absorbance as a percentage of the 24 hr –VN control (depicted by horizontal red line) ± standard error of the means (SEM). Results are from 4 separate experiments. a) Depicts results obtained at 72 hr, while significance (post-hoc t-test) between individual treatments and the 72 hr –VN control or between individual treatments where connected by line is indicated by α (p<0.05) or β (p<0.01). b) Depicts time course results obtained for –VN, VN at 174ng/well (+VN) and ±VN / IGFBP-3 / IGF-I (±VN / 3 / I) treatments while significance between treatments is indicated by * (p<0.05) or # (p<0.01) and are presented below in table 4.3.1.

Table 4.3.1 Summary of statistical analysis for Figure 4.3b

hMSC metabolic activity response to VN/BP-3/I (Time course)		% of -VN @ 24 hr ± SEM	24 hr					48 hr					72 hr				
			-VN	+VN	BP-3/I	VN/BP-3/I	10% FCS	-VN	+VN	BP-3/I	VN/BP-3/I	10% FCS	-VN	+VN	BP-3/I	VN/BP-3/I	10% FCS
24 hr	-VN	100 ± 1.4															
	+VN	150.3 ± 10.3	#														
	BP-3/I	80.5 ± 2.7	#	#													
	VN/BP-3/I	165.1 ± 9.9	#	ns	#												
	10% FCS	155.0 ± 13.9	#	ns	#	ns											
48 hr	-VN	58.5 ± 9.4	#	#	*	#	#										
	+VN	93.4 ± 12.0	ns	#	ns	#	ns	*									
	BP-3/I	52.2 ± 8.7	#	#	#	#	#	ns	#								
	VN/BP-3/I	107.2 ± 10.9	ns	#	*	#	*	#	ns	#							
	10% FCS	105.2 ±3.0	ns	#	#	#	#	#	ns	#	ns						
72 hr	-VN	64.5 ± 8.5	#	#	ns	#	#	ns	ns	ns	#	#					
	+VN	105.8 ± 17.3	ns	*	ns	#	ns	*	ns	#	ns	ns	*				
	BP-3/I	56.2 ± 8.7	#	#	*	#	#	ns	*	ns	#	#	ns	*			
	VN/BP-3/I	119.3 ± 16.7	ns	ns	*	*	ns	#	ns	#	ns	ns	#	ns	#		
	10% FCS	133.0 ± 8.0	#	ns	#	*	ns	#	#	#	ns	#	#	ns	#	ns	

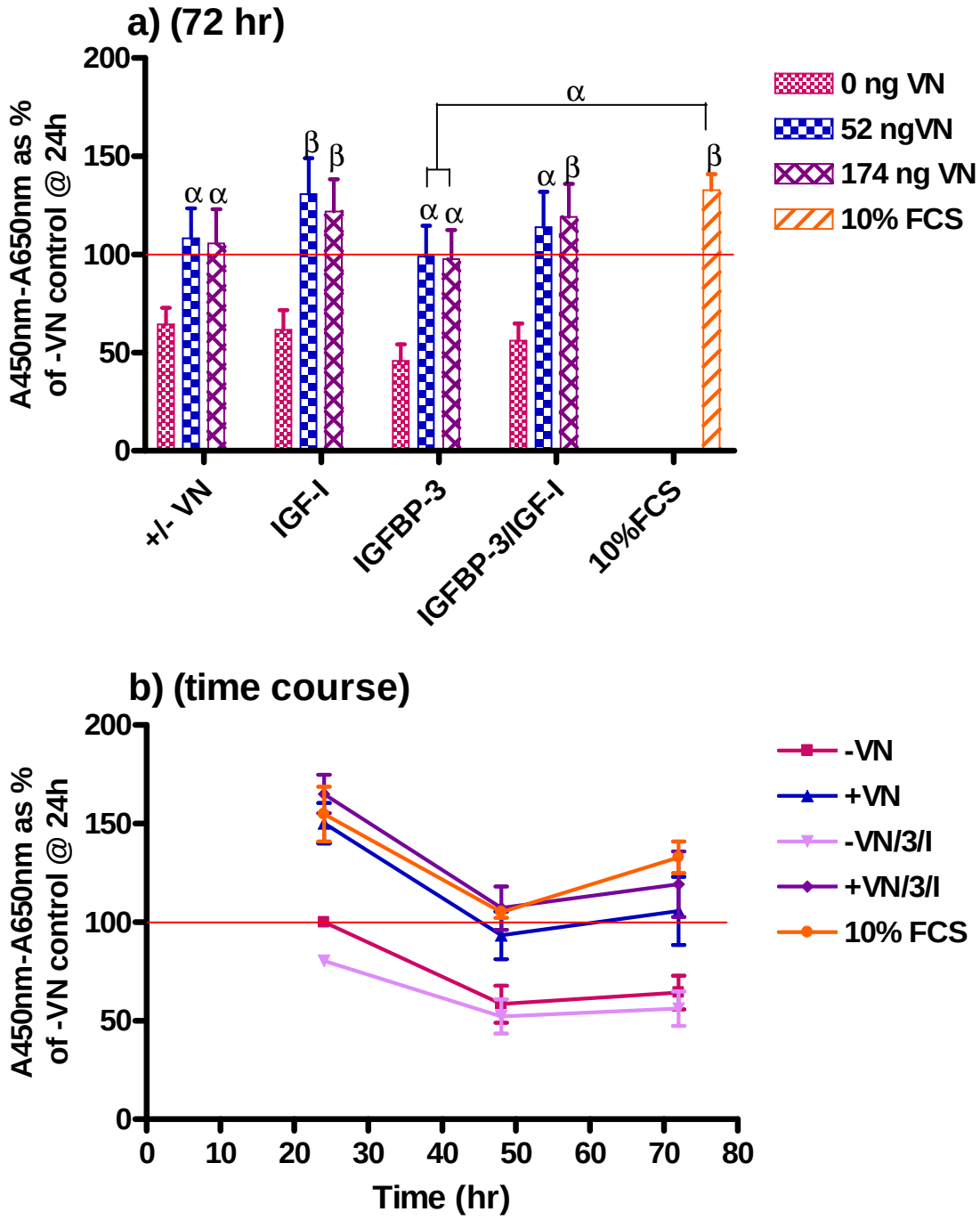

a) (72 hr)
A450nm-A650nm as % of -VN control @ 24h
200
150
100
50
0
α α
β β
α α
α β
α
β
+/- VN
IGF-I
IGFBP-3
IGFBP-3/IGF-I
10%FCS
0 ng VN
52 ngVN
174 ng VN
10% FCS
b) (time course)
A450nm-A650nm as % of -VN control @ 24h
200
150
100
50
0
0 10 20 30 40 50 60 70 80
Time (hr)
-VN
+VN
-VN/3/I
+VN/3/I
10% FCS

Figure 4.3

4.3.4 Effect of solution phase VN and TGF-β_1 on hMSC metabolic activity.

TGF-β_1 and VN have been shown to associate *in vitro* and retain TGF-β_1-mediated cell responses (Schoppet *et al.* 2002). TGF-β_1 is highly expressed in bone tissue (O'Connor-McCourt and Wakefield 1987; Bonewald 1999) and has been shown to elicit a number of effects in hMSCs (Kassem *et al.* 2000; Kveiborg *et al.* 2001b; Spinella-Jaegle *et al.* 2001). I wished to determine if TGF-β_1 together with VN, albeit presented in solution, could influence hMSC metabolic activity. Therefore, while performing the experiments detailed above, I included a selection of TGF-β_1 treatments similar to those used to investigate the influence of the IGFBPs.

We found that by 72 hr the metabolic activity of hMSC cultures exposed to TGF-β_1 in the presence of VN at 52 ng/well, or 174 ng/well (164.5 ± 8.2% (n=11) or 204.5 ± 7.7% (n=12) of the 24 hr –VN control respectively), was not only significantly different to that of the 72 hr –VN control (64.5 ± 8.5% (n=16) of the 24 hr –VN control) (p<0.01) but also higher than that for 1) cultures exposed to VN alone at either 52 ng/well, or 174 ng/well (108.3 ± 15.0% (n=11) or 105.8 ± 17.3% (n=12) of the 24 hr –VN control respectively) (p<0.01) and, significantly, 2) the 10% FCS control (133.0 ± 8.0% (n=24) of the 24 hr –VN control) (p<0.05 or p<0.01 respectively) (Fig 4.4a). In addition the metabolic activity of hMSC cultures exposed to TGF-β_1 alone was equivalent to that found after 72 hr in the –VN control. Taken together these data suggest that when TGF-β_1 is combined with VN a synergistic effect on hMSC metabolic activity is observed after 72 hr.

As previously observed, analysis of the 174 ng/well VN time course data revealed that after 24 hr the metabolic activity of hMSCs exposed to VN / TGF-β_1 (173.1 ± 16.0% (n=12)) was significantly enhanced relative to the 24 hr –VN control (100.0 ± 1.4 % (n=17))(p<0.01). In contrast, the cells treated with TGF-β_1 only (81.4 ± 8.0% (n=17)) possessed significantly lower metabolic activity than the 24 hr –VN control (p<0.01). Unlike any other treatment, however, hMSC metabolic activity did not significantly change between 24 hr and 72 hr in cultures exposed to TGF-β_1 alone. Likewise, cultures exposed to the 174 ng/well VN / TGF-β_1 treatment did not alter metabolic activity between 24 hr and 48 hr (173.1 ± 16.0% (n=12) and 139.1 ± 7.1% (n=12) of the 24 hr –VN control respectively) but did significantly increase hMSC metabolic activity between 48 hr and 72 hr (204.5 ± 7.7% (n=12) of the 24 hr –VN

control) (p<0.01). However, the level of metabolic activity assayed after 72 hr was not significantly different to that assayed after 24 hr (Fig 4.4b and Table 4.4.1).

Figure 4.4. Effect of solution phase VN and TGF-β_1 on hMSC metabolic activity.

Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM or 11.9 nM VN (52ng/well a) or 174ng/well a) & b)) and 1.43nM TGF-β_1 (7ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr b), 48 hr b) or 72 hr a) & b) in a humidified 5% CO_2/95% air atmosphere at 37°C prior to removal of conditioned media, addition of 110 µL of WST-1 / sf-DMEM solution and incubation in the same conditions for a further 2 hr. The absorbance of the coloured formazan cleavage product was quantitated at 450 nm using 650 nm as reference. Results were corrected using data from plate blanks (serum free media and WST-1 alone) and are expressed as the mean corrected absorbance as a percentage of the 24 hr –VN control (depicted by horizontal red line) ± standard error of the means (SEM). Results are from 4 separate experiments. a) Depicts results obtained at 72 hr, while significance (post-hoc t-test) between individual treatments and the 72 hr –VN control or between individual treatments where connected by line is indicated by α (p<0.05) or β (p<0.01). b) Depicts time course results obtained for –VN, VN at 174ng/well (+VN) and ±VN / TGF-β_1 (±VN / TGF-β_1) treatments while significance between treatments is indicated by * (p<0.05) or # (p<0.01) and are presented below in table 4.4.1.

Table 4.4.1 Summary of statistical analysis for Figure 4.4b

hMSC metabolic activity response to VN/TGF-β_1 (Time course)		% of -VN @ 24 hr ± SEM	24 hr					48 hr					72 hr				
			-VN	+VN	TGF-β_1	VN/ TGF-β_1	10% FCS	-VN	+VN	TGF-β_1	VN/ TGF-β_1	10% FCS	-VN	+VN	TGF-β_1	VN/ TGF-β_1	10% FCS
24 hr	-VN	100 ± 1.4															
	+VN	150.3 ± 10.3	#														
	TGF-β_1	81.4 ± 8.0	*	#													
	VN/ TGF-β_1	173.1 ± 16.0	#	ns	#												
	10% FCS	155.0 ± 13.9	#	ns	#	ns											
48 hr	-VN	58.5 ± 9.4	#	#	ns	#	#										
	+VN	93.4 ± 12.0	ns	#	ns	#	*	*									
	TGF-β_1	66.8 ± 4.5	#	#	ns	#	#	ns	*								
	VN/TGF-β_1	139.1 ± 7.1	#	ns	#	ns	ns	#	#	#							
	10% FCS	105.2 ± 3.0	ns	#	#	#	#	#	ns	#	#						
72 hr	-VN	64.5 ± 8.5	#	#	ns	#	#	ns	ns	ns	#	#					
	+VN	105.8 ± 17.3	ns	*	ns	#	ns	*	ns	*	ns	ns	*				
	TGF-β_1	67.5 ± 5.6	#	#	ns	#	#	ns	*	ns	#	#	ns	*			
	VN/ TGF-β_1	204.5 ± 7.7	#	#	#	ns	*	#	#	#	#	#	#	#	#		
	10% FCS	133.0 ± 8.0	#	ns	#	*	ns	#	#	#	ns	#	#	ns	#	#	

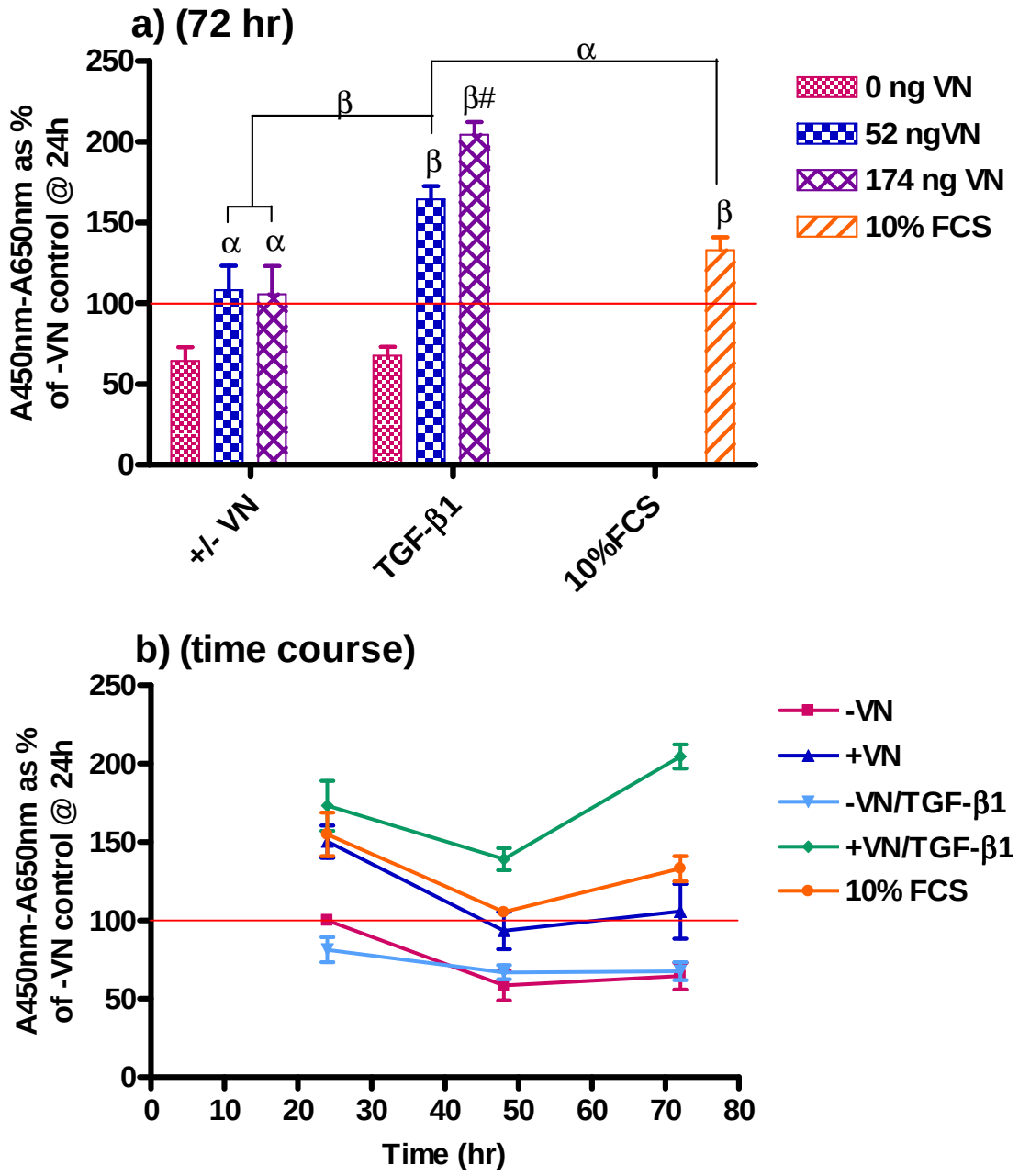

a) (72 hr)
A450nm-A650nm as % of -VN control @ 24h
250
200
150
100
50
0
α
β
β#
β
α
α
β
+/- VN
TGF-β1
10%FCS
0 ng VN
52 ngVN
174 ng VN
10% FCS
b) (time course)
A450nm-A650nm as % of -VN control @ 24h
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
Time (hr)
-VN
+VN
-VN/TGF-β1
+VN/TGF-β1
10% FCS

Figure 4.4

4.3.5 Effect of solution phase VN, IGFBP-5 and IGF-I on hMSC total protein.

While measurement of metabolic activity may provide an indication of the effect of a particular treatment on cell function at the time of measurement, it does not necessarily give an indication as to any cumulative effects that the treatment may cause such as protein synthesis or cell proliferation. Therefore in order to broadly determine whether various combinations of VN, IGFBP-3 or -5 and IGF-I or TGF-β_1 may facilitate any cumulative effect on hMSCs, I decided to coincidently measure the relative total protein of hMSCs in the assays detailed above. hMSC monolayer cultures stained with the vital dye crystal violet demonstrated that after 72 hr there was no difference in the total protein of hMSCs exposed to VN at either 52 ng/well (3.55 nM) or 174 ng/well (11.9 nM) in any treatment (Fig 4.5a). Thus the VN concentration had no effect on the total protein accumulation within the monolayers of hMSCs exposed to any of the treatments, except when absent altogether. However, VN alone at 174 ng/well (173.7 ± 21.8% (n=12) of 24 hr –VN control) yielded a significantly higher total protein accumulation after 72 hr than hMSCs exposed to sf-DMEM alone (72 hr –VN control) (115.3 ± 11.1% (n=12) of 24 hr –VN control) (p<0.05). Interestingly, VN at 52 ng/well (162.5 ± 20.0% (n=12) of the 24 hr –VN control), while elevated, did not support significantly higher protein accumulation compared to the 72 hr –VN control. Where IGF-I was present with VN at 52 ng/well or 174 ng/well (206.9 ± 26.1% (n=12) or 198.6 ± 19.8% (n=12) of 24 hr –VN control respectively) significantly more protein was present after 72 hr than the 72 hr –VN control (p<0.01). Similarly, hMSCs exposed to IGFBP-5 alone, but in the presence of VN at 52 ng/well or 174 ng/well (168.0 ± 21.8% (n=12) or 194.3 ± 26.8% (n=12) of the 24 hr –VN control respectively), also displayed significantly elevated levels of total protein compared to the 72 hr –VN control (p<0.05), as did cultures exposed to IGFBP-5 / IGF-I with VN at 52 ng/well or 174 ng/well (209.0 ± 28.4% (n=12) or 232.9 ± 32.0% (n=12) of 24 hr –VN control respectively) (p<0.01). Interestingly, of the treatments containing VN, only cultures exposed to either VN alone at 52 ng/well or VN at 52 ng/well with IGFBP-5 yield significantly less total protein than the 72 hr 10% FCS control (241.5 ± 21.8% (n=21) of the –VN control) (p<0.05).

Analysis of the VN at 174 ng/well time course data showed that after 24 hr the total protein of cultures exposed to VN alone, VN / IGFBP-5 / IGF-I or 10% FCS (142.6

± 13.6% (n=12), 173.3 ± 18.4% (n=12) or 156.4 ± 11.5% (n=24) of the 24 hr –VN control respectively) was significantly higher than the 24 hr –VN control (100.0 ± 9.5 % (n=12)) ($p<0.05$, $p<0.01$ and $p<0.01$ respectively). However, total protein measured in hMSC cultures exposed to IGFBP-5 / IGF-I (82.2 ± 8.2% (n=12) of the 24 hr –VN control) or the 24 hr –VN control (Fig 4.5b and Table 4.5.1) were statistically equivalent. While all treatments increased hMSC total protein over 72 hr, none of the increases were significant with the exception of the 10% FCS treatment, which produced a significant increase in total protein between 24 hr and 72 hr (156.4 ± 11.5% (n=24) and 241.5 ± 21.8% (n=21) of the 24 hr –VN control respectively)($p<0.01$). Taken together these data indicate that in this system VN is primarily responsible for influencing the total protein level of hMSCs after 72 hr and that the addition of IGF-I elevates this. The addition of IGFBP-5 together with IGF-I does not increase total protein levels over that assayed for IGF-I together with VN.

Figure 4.5. Effect of solution phase VN, IGFBP-5 and IGF-I on hMSC total protein. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM or 11.9 nM VN (52 ng/well or 174 ng/well), 11.9 nM IGFBP-5 (70 ng/well) and 11.9 nM IGF-I (17.4 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr b), 48 hr b) or 72 hr a) & b) in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and the crystal violet extracted with 10% acetic acid. The absorbance of each well was then quantitated at 595 nm. Results were corrected using data from plate blanks (10% acetic acid alone) and are expressed as the mean corrected absorbance as a percentage of the 24 hr –VN control (depicted by horizontal red line) ± standard error of the means (SEM). Results are from 4 separate experiments. a) Depicts results obtained at 72 hr, while significance (post-hoc t-test) between individual treatments and the 72 hr –VN control or between individual treatments where connected by line is indicated by α (p<0.05) or β (p<0.01). b) Depicts time course results obtained for –VN, VN at 174ng/well (+VN) and ±VN / IGFBP-5 / IGF-I (±VN / 5 / I) treatments while significance between treatments is indicated by * (p<0.05) or # (p<0.01) and are presented below in table 4.5.1.

Table 4.5.1 Summary of statistical analysis for Figure 4.5b

hMSC total protein assay VN/BP-5/I (Time course)		% of -VN @ 24 hr ± SEM	24 hr					48 hr					72 hr				
			-VN	+VN	BP-5/I	VN/BP-5/I	10% FCS	-VN	+VN	BP-5/I	VN/BP-5/I	10% FCS	-VN	+VN	BP-5/I	VN/BP-5/I	10% FCS
24 hr	-VN	100 ± 9.5															
	+VN	142.6 ± 13.6	*														
	BP-5/I	82.2 ± 8.2	ns	#													
	VN/BP-5/I	173.3 ± 18.4	#	ns	#												
	10% FCS	156.4 ± 11.5	#	ns	#	ns											
48 hr	-VN	103.4 ± 11.9	ns	*	ns	#	#										
	+VN	140.4 ± 17.5	ns	ns	#	ns	ns	ns									
	BP-5/I	95.4 ± 8.4	ns	#	ns	#	#	ns	*								
	VN/BP-5/I	185.1 ± 24.9	#	ns	#	ns	ns	#	ns	#							
	10% FCS	194.2 ± 17.2	#	*	#	ns	ns	#	ns	#	ns						
72 hr	-VN	115.3 ± 11.1	ns	ns	*	*	*	ns	ns	ns	*	#					
	+VN	173.7 ± 21.8	#	ns	#	ns	ns	#	ns	#	ns	ns	*				
	BP-5/I	108.9 ± 16.8	ns	ns	ns	*	*	ns	ns	ns	*	#	ns	*			
	VN/BP-5/I	232.9 ± 32.0	#	*	#	ns	*	#	*	#	ns	ns	#	ns	#		
	10% FCS	241.5 ± 21.8	#	#	#	*	#	#	#	#	ns	ns	#	ns	#	ns	

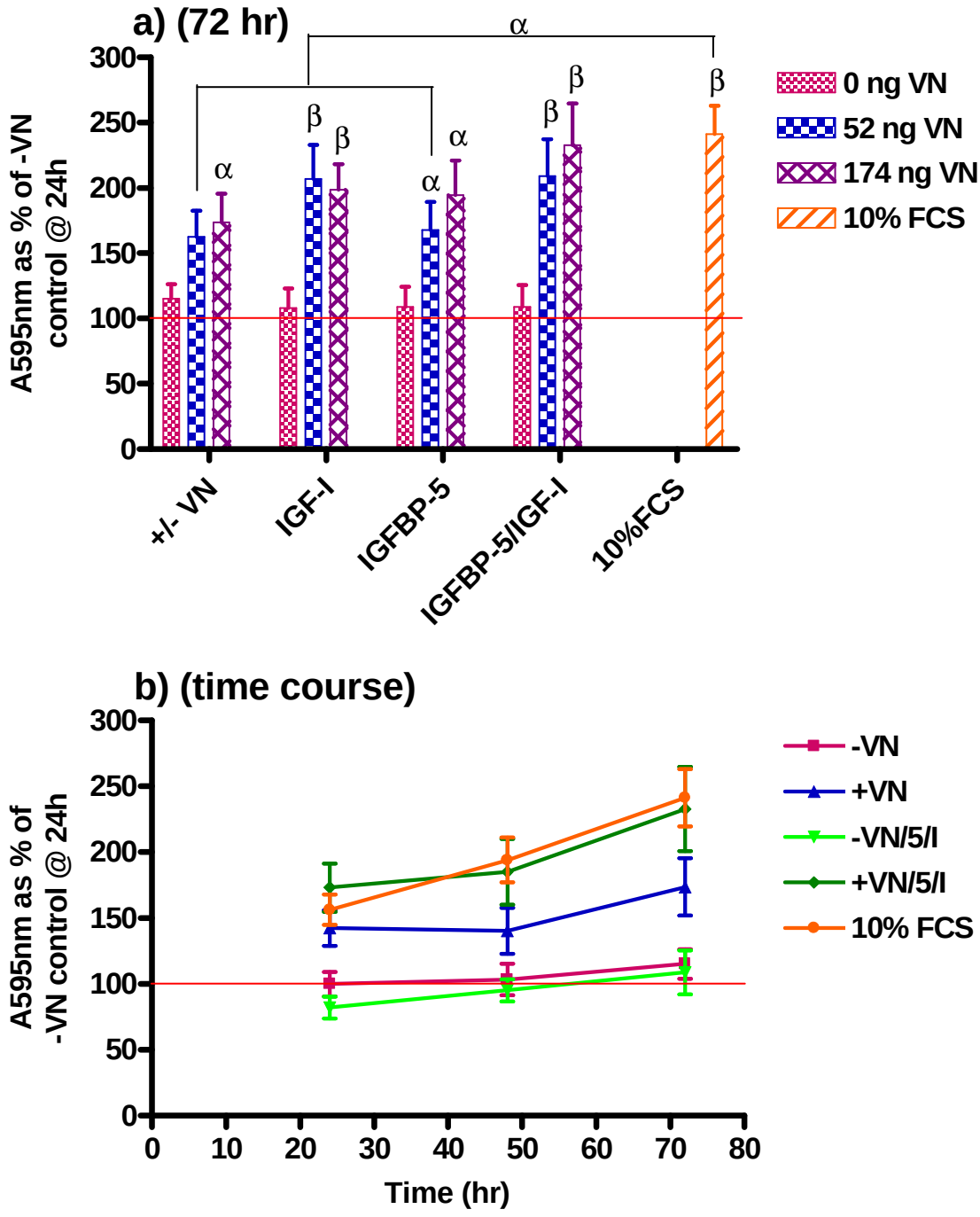

a) (72 hr)
A595nm as % of -VN control @ 24h
300
250
200
150
100
50
0
α
β
+/- VN
IGF-I
IGFBP-5
IGFBP-5/IGF-I
10%FCS
0 ng VN
52 ng VN
174 ng VN
10% FCS
b) (time course)
A595nm as % of -VN control @ 24h
0
10
20
30
40
50
60
70
80
Time (hr)
-VN
+VN
-VN/5/I
+VN/5/I
10% FCS

Figure 4.5

4.3.6 Effect of solution phase VN, IGFBP-3 and IGF-I on hMSC total protein.

Similar to the results obtained for the metabolic activity assays, the total protein response of hMSCs exposed to the various treatments incorporating either IGFBP-3 or IGFBP-5 were almost identical. Specifically, I found that after 72 hr hMSCs exposed to IGFBP-3 in the presence of VN at 52 ng/well (152.2 ± 17.3% (n=12) of the 24 hr –VN control) did not have significantly more total protein compared to the 72 hr –VN control (115.3 ± 11.1% (n=12) of the 24 hr –VN control) and had significantly less total protein than the 72 hr 10% FCS control (241.5 ± 21.8% (n=12) of the 24 hr –VN control) (p<0.01) (Fig 4.6a). In contrast, hMSCs exposed to IGFBP-3 in the presence of VN at 174 ng/well (164.0 ± 17.1% (n=12) of the 24 hr –VN control) had significantly more total protein compared to the 72 hr –VN control (p<0.05), as did cultures exposed to IGFBP-3 / IGF-I with VN at 52 ng/well or 174 ng/well (209.5 ± 26.8% (n=12) or 237.3 ± 29.9% (n=12) of 24 hr –VN control respectively) (p<0.01). However, the hMSC response to the IGFBP-3 / 174 ng of VN treatment was also significantly less than the response to 10% FCS (p<0.05) after 72 hr, whereas there was no statistical difference between either of the IGFBP-3 / IGF-I responses and the 72 hr 10% FCS control. Interestingly, I found that hMSCs exposed to IGFBP-3 / IGF-I in the presence of VN at 174 ng/well accumulated significantly more total protein than IGFBP-3 with VN at either 52 ng/well or 174 ng/well or than VN alone at 52 ng/well (p<0.05).

Analysis of the response of cells to VN at 174 ng/well over time showed that after 24 hr the total protein of the cultures exposed to VN / IGFBP-3 / IGF-I (182.2 ± 20.1% (n=12) of the 24 hr –VN control) was significantly higher than the 24 hr –VN control (100.0 ± 9.5 % (n=12)) (p<0.01). However, there was no difference in total protein between hMSC cultures exposed to IGFBP-3 / IGF-I (102.8 ± 9.1% (n=12) of the 24 hr –VN control) or the 24 hr –VN control (Fig 4.6b and Table 4.6.1). Paralleling the responses found with cultures exposed to IGFBP-5, treatments containing IGFBP-3 increased hMSC total protein over 72 hr although these increases were not statistically significant with the exception, as described above, of the 10% FCS treatment which produced a significant increase in total protein between 24 hr and 72 hr. These data suggest that there is little effect if any on the total protein content of cultures exposed to either IGFBP-3 or IGFBP-5, regardless of whether VN or IGF-I or both are present or not.

Figure 4.6. Effect of solution phase VN, IGFBP-3 and IGF-I on hMSC total protein. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM or 11.9 nM VN (52 ng/well or 174 ng/well), 11.9 nM IGFBP-3 (70 ng/well) and 11.9 nM IGF-I (17.4 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr b), 48 hr b) or 72 hr a) & b) in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and the crystal violet extracted with 10% acetic acid. The absorbance of each well was then quantitated at 595 nm. Results were corrected using data from plate blanks (10% acetic acid alone) and are expressed as the mean corrected absorbance as a percentage of the 24 hr –VN control (depicted by horizontal red line) ± standard error of the means (SEM). Results are from 4 separate experiments. a) Depicts results obtained at 72 hr, while significance (post-hoc t-test) between individual treatments and the 72 hr –VN control or between individual treatments where connected by line is indicated by α (p<0.05) or β (p<0.01). b) Depicts time course results obtained for –VN, VN at 174ng/well (+VN) and ±VN / IGFBP-3 / IGF-I (±VN / 3 / I) treatments while significance between treatments is indicated by * (p<0.05) or # (p<0.01) and are presented below in table 4.6.1.

Table 4.6.1 Summary of statistical analysis for Figure 4.6b

hMSC total protein assay VN/BP-3/I (Time course)		% of -VN @ 24 hr ± SEM	24 hr					48 hr					72 hr				
			-VN	+VN	BP-3/I	VN/BP-3/I	10% FCS	-VN	+VN	BP-3/I	VN/BP-3/I	10% FCS	-VN	+VN	BP-3/I	VN/BP-3/I	10% FCS
24 hr	-VN	100 ± 9.5															
	+VN	142.6 ± 13.6	*														
	BP-3/I	102.8 ± 9.1	ns	*													
	VN/BP-3/I	182.2 ± 20.1	#	ns	#												
	10% FCS	156.4 ± 11.5	#	ns	#	ns											
48 hr	-VN	103.4 ± 11.9	ns	*	ns	#	#										
	+VN	140.4 ± 17.5	ns	ns	ns	ns	ns	ns									
	BP-3/I	106.1 ± 7.1	ns	*	ns	#	#	ns	ns								
	VN/BP-3/I	200.4 ± 27.0	#	ns	#	ns	ns	#	ns	#							
	10% FCS	194.2 ± 17.2	#	*	#	ns	ns	#	ns	#	ns						
72 hr	-VN	115.3 ± 11.1	ns	ns	ns	#	*	ns	ns	ns	#	#					
	+VN	173.7 ± 21.8	#	ns	#	ns	ns	#	ns	#	ns	ns	*				
	BP-3/I	131.1 ± 17.6	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns			
	VN/BP-3/I	237.3 ± 29.9	#	*	#	ns	#	#	*	#	ns	ns	#	ns	#		
	10% FCS	241.5 ± 21.8	#	#	#	ns	#	#	#	#	ns	ns	#	ns	#	ns	

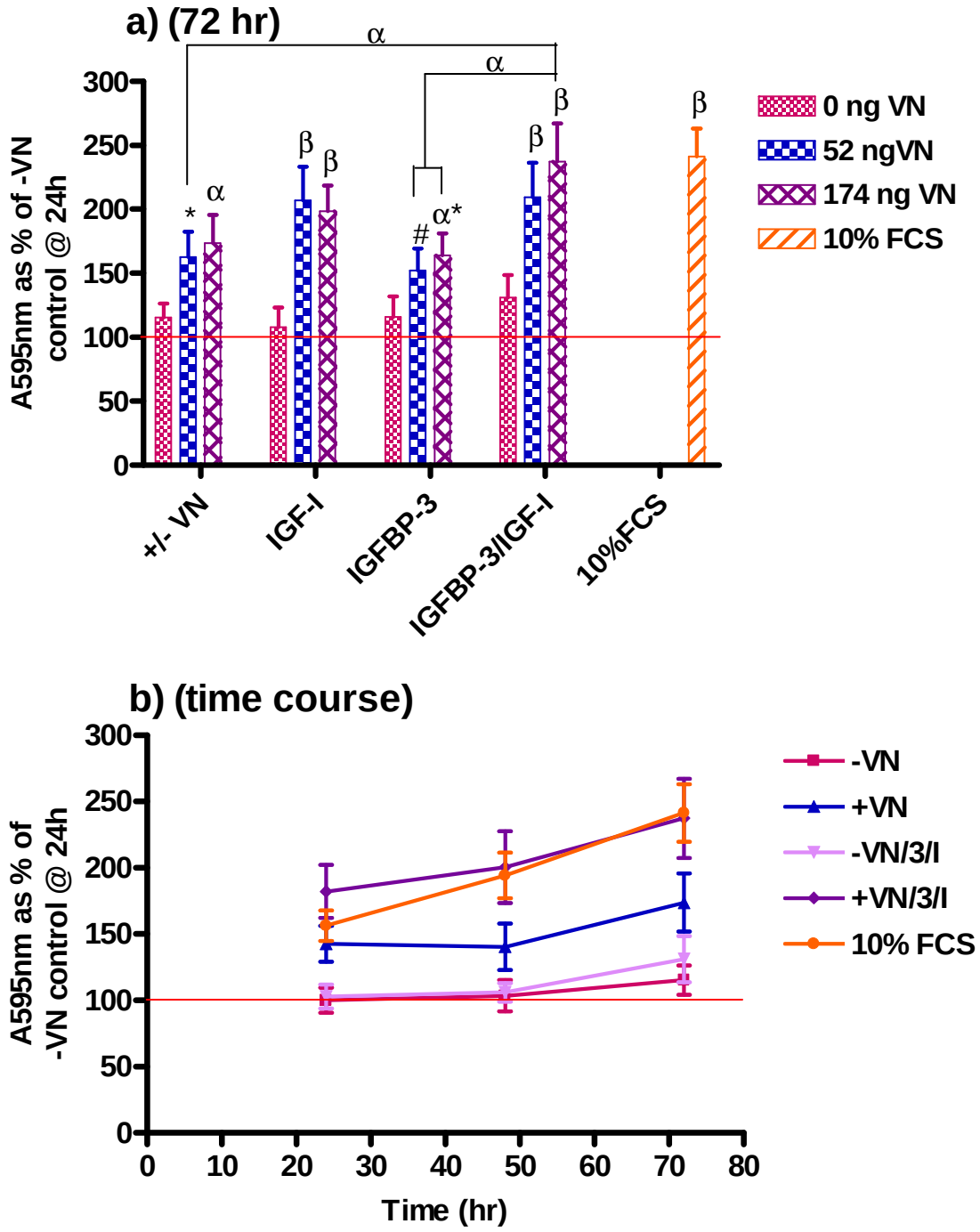

a) (72 hr)
A595nm as % of -VN control @ 24h
300
250
200
150
100
50
0
α
α
β
β
β
β
β
*
α
#
α*
+/- VN
IGF-I
IGFBP-3
IGFBP-3/IGF-I
10%FCS
0 ng VN
52 ngVN
174 ng VN
10% FCS
b) (time course)
A595nm as % of -VN control @ 24h
300
250
200
150
100
50
0
0
10
20
30
40
50
60
70
80
Time (hr)
-VN
+VN
-VN/3/I
+VN/3/I
10% FCS

Figure 4.6

4.3.7 Effect of solution phase VN and TGF-β_1 on hMSC total protein.

As discussed above, TGF-β_1 can associate with VN and therefore I wished to investigate the effect of TGF-β_1 on hMSC total protein over 72 hr. I found that after 72 hr hMSCs exposed to TGF-β_1 in the presence of VN at 52 ng/well (184.9 ± 22.7% (n=12) of the 24 hr –VN control) accumulated significantly more total protein than either the 72 hr –VN control (115.3 ± 11.1% (n=12) of the 24 hr –VN control) or hMSCs cultured in the presence of TGF-β_1 without VN (122.6 ± 14.7% (n=12) of the 24 hr -VN control) (p<0.05). Similarly, hMSCs exposed to TGF-β_1 in the presence of VN at 174 ng/well (223.8 ± 26.9% (n=12) of the 24 hr –VN control) had significantly more protein than either the 72 hr –VN control or the TGF-β_1 only treatment (p<0.01) (Fig 4.7a). Neither TGF-β_1 / VN treatments resulted in statistically significant differences in total protein responses compared to the 72 hr 10% FCS control (241.5 ± 21.8% (n=21) of the 24 hr –VN control), nor were they statistically different to the results of either of the VN only treatments after 72 hr. In a similar manner, hMSC total protein in TGF-β_1 only was not different to that for the 72 hr –VN control but had significantly less total protein than the 72 hr 10% FCS control (p<0.01) (Fig 4.7a). Taken together, these data indicate that while culturing of hMSCs in the presence of TGF-β_1 and VN in solution can increase hMSC total protein compared to VN alone, this increase is transient and is not significant after 72 hr.

The 174 ng/well time course data showed that after 24 hr the total protein of cultures exposed to VN / TGF-β_1 (156.1 ± 14.9% (n=12) of the 24 hr –VN control) was significantly higher than the 24 hr –VN control (100.0 ± 9.5 % (n=12)) (p<0.01). However, there was no difference in total protein between hMSC cultures exposed to TGF-β_1 (93.3 ± 10.1% (n=12) of the 24 hr –VN control) and the 24 hr –VN control. In contrast to the IGFBP-5 and IGFBP-3 data presented above, TGF-β_1, in the presence of VN, significantly increased hMSC total protein between 24 hr and 72 hr (223.8 ± 26.9% (n=12) or the 24 hr –VN control) (p<0.05) (Fig 4.7b and Table 4.7.1). This indicates that TGF-β_1 together with VN can stimulate an increase in hMSC total protein over 72 hr and this seems to be in contrast (at least statistically) to VN / IGFBP-5 / IGF-I or IGFBP-3 / IGF-I.

Figure 4.7. Effect of solution phase VN and TGF-β_1 on hMSC total protein.
Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM or 11.9 nM VN (52 ng/well or 174 ng/well) and 1.43 nM TGF-β_1 (7 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr b), 48 hr b) or 72 hr a) & b) in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and the crystal violet extracted with 10% acetic acid. The absorbance of each well was then quantitated at 595 nm. Results were corrected using data from plate blanks (10% acetic acid alone) and are expressed as the mean corrected absorbance as a percentage of the 24 hr –VN control (depicted by horizontal red line) ± standard error of the means (SEM). Results are from 4 separate experiments. a) Depicts results obtained at 72 hr, while significance (post-hoc t-test) between individual treatments and the 72 hr –VN control or between individual treatments where connected by line is indicated by α (p<0.05) or β (p<0.01). b) Depicts time course results obtained for –VN, VN at 174ng/well (+VN) and ±VN / TGF-β_1 treatments while significance between treatments is indicated by * (p<0.05) or # (p<0.01) and are presented below in table 4.7.1.

Table 4.7.1 Summary of statistical analysis for Figure 4.7b

hMSC total protein assay VN/TGF-β_1 (Time course)		% of -VN @ 24 hr ± SEM	24 hr					48 hr					72 hr				
			-VN	+VN	TGF-β_1	VN/ TGF-β_1	10% FCS	-VN	+VN	TGF-β_1	VN/ TGF-β_1	10% FCS	-VN	+VN	TGF-β_1	VN/ TGF-β_1	10% FCS
24 hr	-VN	100 ± 9.5															
	+VN	142.6 ± 13.6	*														
	TGF-β_1	93.3 ± 10.1	ns	#													
	VN/ TGF-β_1	156.1 ± 14.9	#	ns	#												
	10% FCS	156.4 ± 11.5	#	ns	#	ns											
48 hr	-VN	103.4 ± 11.9	ns	*	ns	*	#										
	+VN	140.4 ± 17.5	ns	ns	*	ns	ns	ns									
	TGF-β_1	105.2 ± 10.7	ns	*	ns	*	#	ns	ns								
	VN/ TGF-β_1	175.8 ± 22.5	#	ns	#	ns	ns	#	ns	*							
	10% FCS	194.2 ± 17.2	#	*	#	ns	ns	#	ns	#	ns						
72 hr	-VN	115.3 ± 11.1	ns	ns	ns	*	*	ns	ns	ns	*	#					
	+VN	173.7 ± 21.8	#	ns	#	ns	ns	#	ns	*	ns	ns	*				
	TGF-β_1	122.6 ± 14.7	ns	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns			
	VN/ TGF-β_1	223.8 ± 26.9	#	*	#	*	*	#	*	#	ns	ns	#	ns	#		
	10% FCS	241.5 ± 21.8	#	#	#	#	#	#	#	#	ns	ns	#	ns	#	ns	

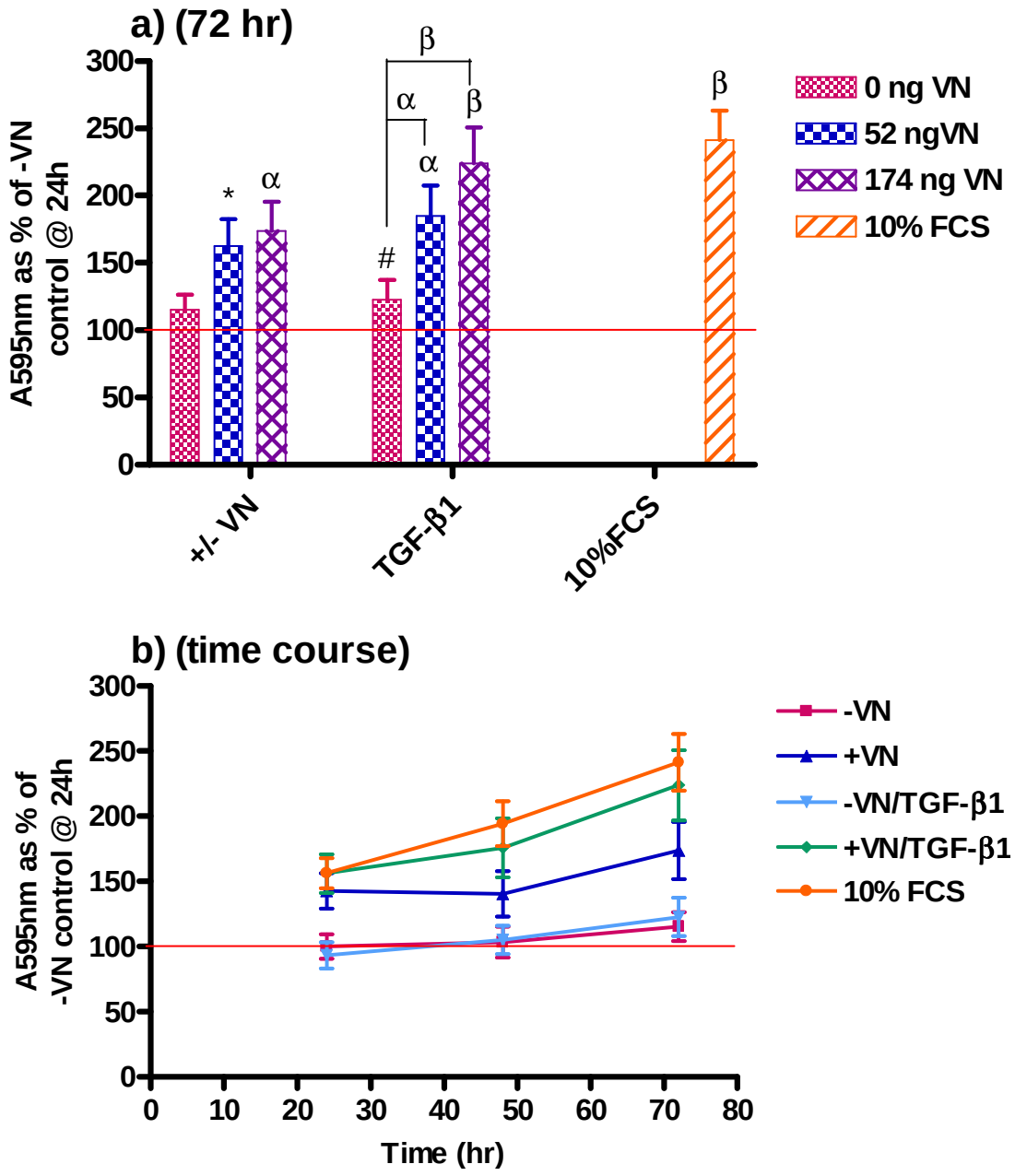

a) (72 hr)
A595nm as % of -VN control @ 24h
300
250
200
150
100
50
0
*
α
#
α
β
α
β
β
+/- VN
TGF-β1
10%FCS
0 ng VN
52 ngVN
174 ng VN
10% FCS
b) (time course)
A595nm as % of -VN control @ 24h
0
10
20
30
40
50
60
70
80
Time (hr)
-VN
+VN
-VN/TGF-β1
+VN/TGF-β1
10% FCS

Figure 4.7

4.3.8 Time dependent effect of IGF-I and TGF-β_1 on hMSC culture morphology.

Prior to solubilisation of the crystal violet stain in the total protein assays described above I inspected the stained cell monolayers under the microscope and noticed distinct morphological differences between some of the treatments; consequently each well was photographed in order to assess the impact of each treatment on hMSC culture morphology. Initially few cells remained attached in cultures which had been exposed to treatments without VN, after 24 hr (data not shown). This suggested that in this system VN was critical for facilitating hMSC attachment to the culture surface. In contrast hMSC cultures exposed to treatments containing VN at either 52 ng/well (Fig 4.8a.1 – a.8) or 174 ng/well (not shown), were relatively uniformly distributed throughout each well with most cells appearing spread and well attached. Cells cultured in 10% FCS appeared to have more homogeneous nuclei and cytoskeletal structure (Fig 4.8a.7). Cells in the VN containing treatments on the other hand appeared a little more heterogeneous in their cytoskeletal architecture. In addition, there was some evidence of slightly more intense crystal violet staining of isolated patches throughout the wells (Fig 4.8a.1-6 and 4.8a.8). After 48 hr distinct differences in culture morphology began to appear. Specifically, hMSC cultures exposed to VN at 52 ng/well and in the presence of either IGF-I (with or without either of the IGFBPs) or TGF-β_1 began to contract and aggregate exposing large gaps/holes in the monolayer (Fig 4.8b.2, 4, 6 & 8). However, hMSC cultures which were exposed to 52 ng/well of VN in the presence of either of the IGFBPs developed only relatively small gaps/holes in their respective monolayers (Fig 4.8b.3 & 5). Cultures exposed to VN alone at 52 ng/well and 10% FCS remained relatively evenly distributed throughout their respective wells with little or no contraction (Fig 4.8b.1 & 7). After 72 hr hMSC cultures exposed to either IGF-I (with or without either of the IGFBPs) or TGF-β_1, together with VN at 52 ng/well had further contracted, leaving large areas of culture surface devoid of cells (Fig 4.8c.2, 4, 6 and 8). hMSC cultures exposed to VN at 52 ng/well and IGFBP-3, and to a slightly lesser extent IGFBP-5, had also started to develop similar but much smaller gaps/holes in their respective monolayers (Fig 4.8c.3 & 5). However, after 72 hr, hMSC cultures exposed to VN alone at 52 ng/well or 10% FCS had still not formed any substantial gaps/holes similar to those observed for cultures in the presence of either IGF-I or TGF-β_1 (Fig 4.8c.1 & 7). These data indicate that in this

system IGF-I and TGF-β_1 can induce time dependent aggregation and contraction of hMSC cultures.

4.3.9 Effect of VN concentration on IGF-I and TGF-β_1 mediated hMSC culture aggregation.

In contrast to the morphological changes observed when hMSC cultures were exposed to VN at 52 ng/well in the presence of IGF-I or TGF-β_1, there was no evidence of any substantial contraction of hMSC cultures exposed to VN at 174 ng/well after 48 hr (data not shown). Indeed, even after 72 hr hMSC cultures exposed to either IGF-I or TGF-β_1 in the presence of VN at 174 ng/well had retained a similar confluent morphology in their monolayers (Fig 4.9a.2, 4, 6 & 8).

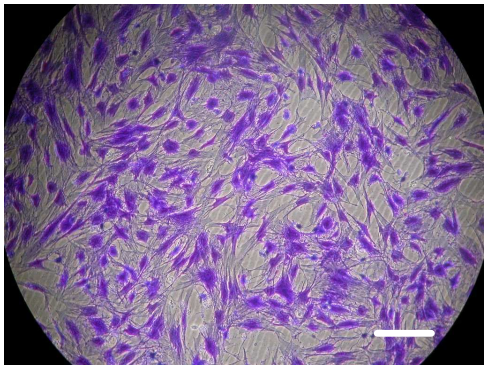
Furthermore, hMSC cultures exposed to IGFBP-3 or -5 in the presence of VN at 174 ng/well also did not develop any clear gaps / holes in the monolayers (Fig 4.9a.3 & 5) unlike similarly treated cultures exposed VN at 52 ng/well as detailed above. Similarly, the hMSC cultures exposed to VN alone at 174 ng/well appeared confluent with no discernable aggregation of cells and no obvious gap / hole formation, as was the case for cells exposed to VN at 52 ng/well at the same time point (Fig 4.9a.1). Thus, VN at the higher concentration appears to have an antagonistic effect on the time dependent aggregation / contraction of hMSC cultures in response to IGF-I and TGF-β_1 detailed above.

4.3.10 Donor effect on IGF-I and TGF-β_1 mediated hMSC culture aggregation.

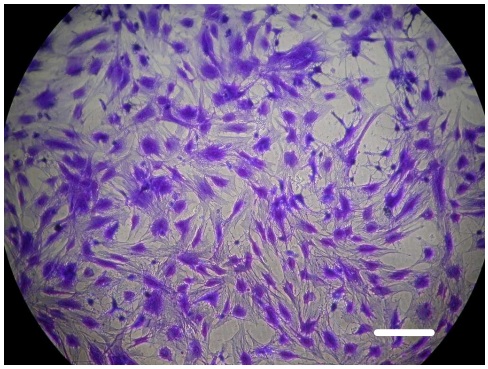
In order to demonstrate that this effect was not a donor specific response I captured images of each well from the total protein assays detailed above (section 4.2.6 & 4.2.7). A comparison of hMSC cultures, sourced from 3 separate donors, exposed to identical treatments detailed above, revealed that there was no donor specific effect. Representative images of hMSCs from each donor exposed to VN at either 52 ng/well or 174 ng/well in the presence or absence of IGFBP-5 with or without IGF-I exhibited similar responses to each treatment between donors (Fig 4.10).

Figure 4.8a. Effect of IGF-I and TGF-β_1 on hMSC culture morphology after 24 hr. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of 3.55 nM VN (52 ng/well) and 11.9 nM IGFBP-3 or -5 (70 ng/well) and 11.9 nM IGF-I (17.4ng/well) or 1.43nM TGF-β_1 (7 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 24 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and digital images of each well captured at X100 magnification. Bar = 200µm.

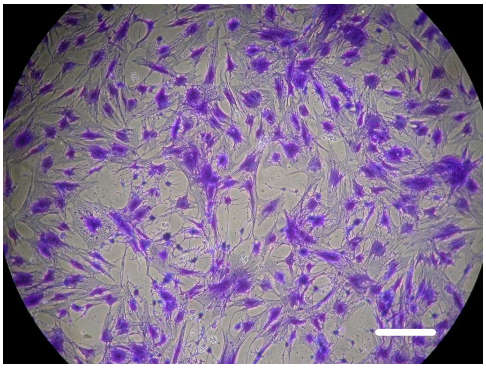
x100



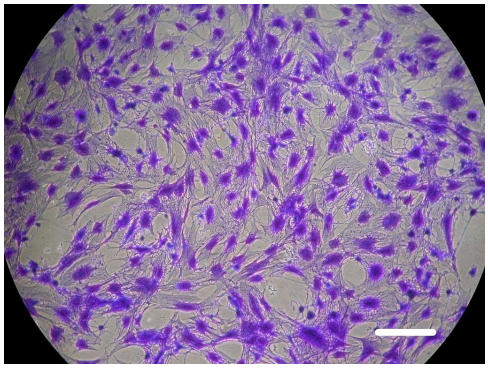
a.1 VN



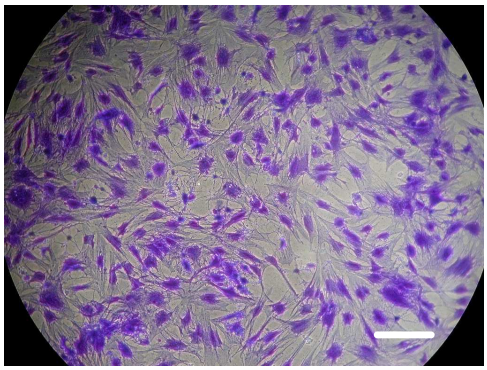
a.2 VN/IGF-I



a.3 VN/IGFBP-3



a.4 VN/IGFBP-3/IGF-I



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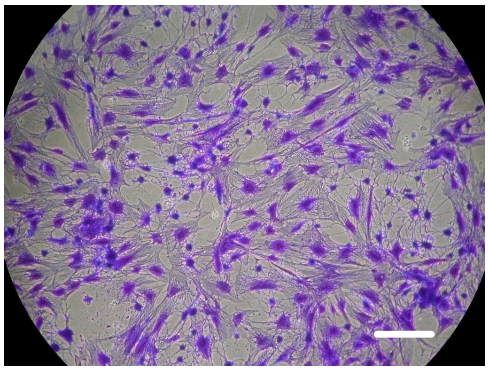
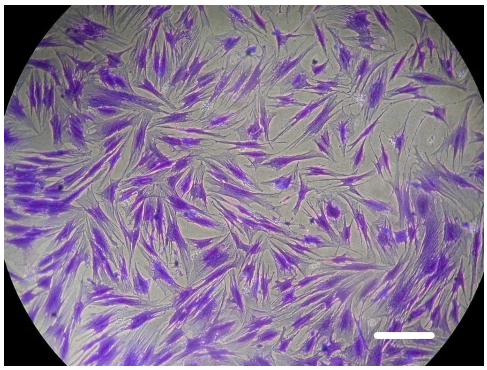
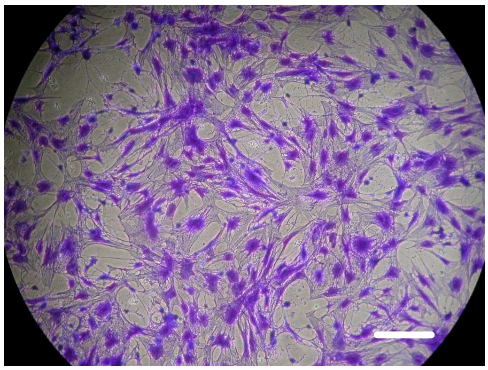


a.6 VN/IGFBP-5/IGF-I



a.7 10% FCS

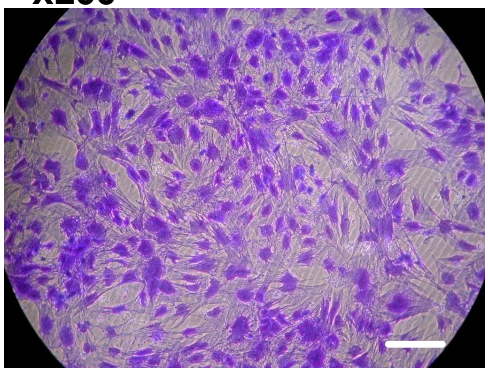


a.8 VN/TGF-ß$_1$

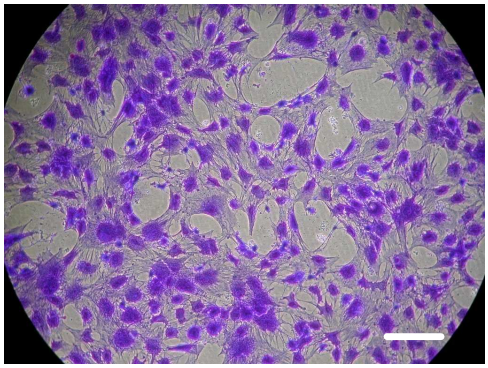
Figure 4.8a

Figure 4.8b. Effect of IGF-I and TGF-β_1 on hMSC culture morphology after 48 hr. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of 3.55 nM VN (52 ng/well) and 11.9 nM IGFBP-3 or -5 (70 ng/well) and 11.9 nM IGF-I (17.4ng/well) or 1.43nM TGF-β_1 (7 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 48 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and digital images of each well captured at X100 magnification. Bar = 200µm.

x100



b.1 VN



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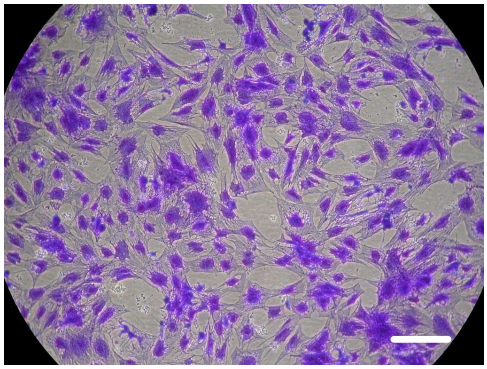
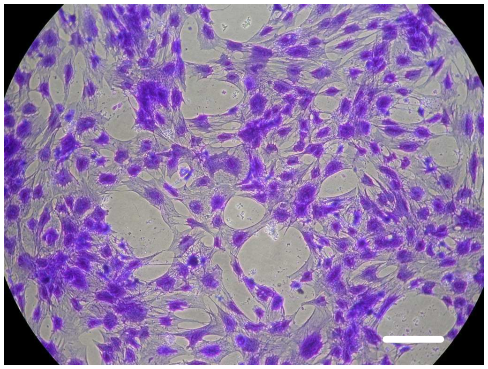


b.3 VN/IGFBP-3



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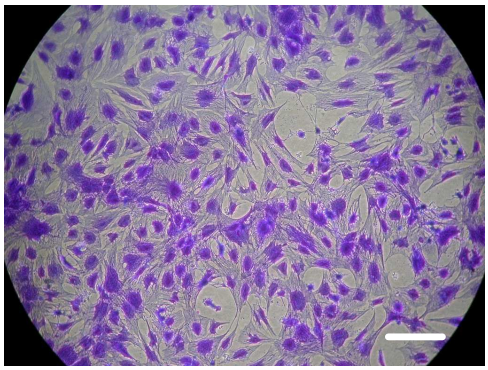

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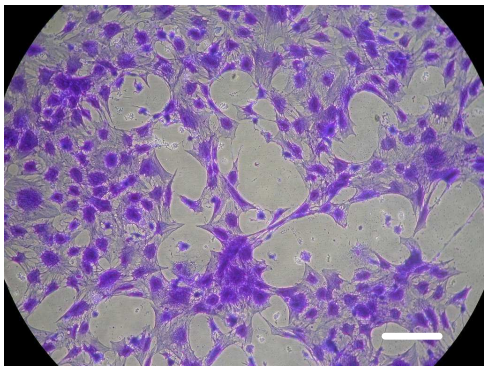

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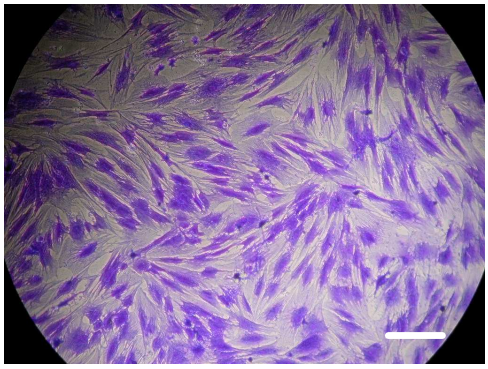
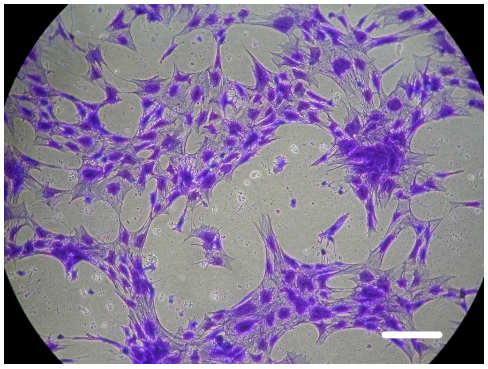


b.7 10% FCS

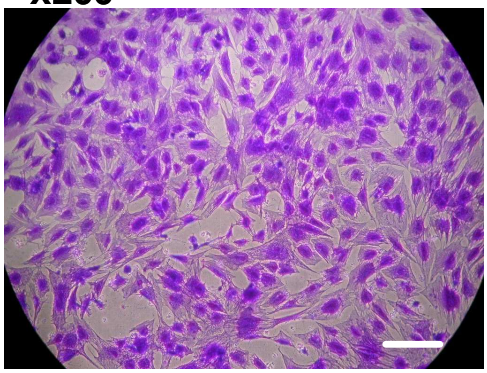


b.8 VN/TGF-ß$_1$

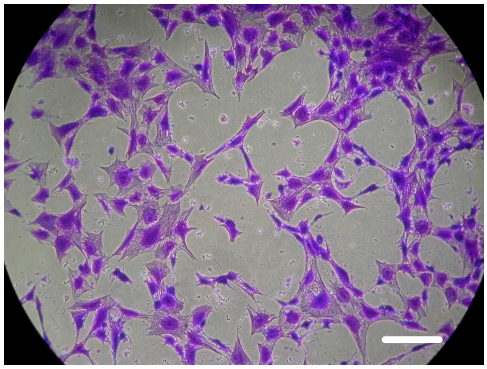
Figure 4.8b

Figure 4.8c. Effect of IGF-I and TGF-β_1 on hMSC culture morphology after 72 hr. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of 3.55 nM VN (52 ng/well) and 11.9 nM IGFBP-3 or -5 (70 ng/well) and 11.9 nM IGF-I (17.4ng/well) or 1.43nM TGF-β_1 (7 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 72 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and digital images of each well captured at X100 magnification. Bar = 200µm.

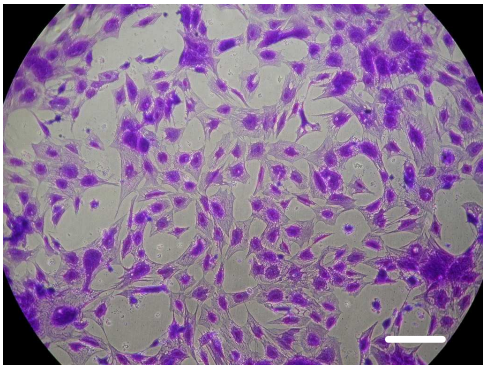
x100



c.1 VN



c.2 VN/IGF-I



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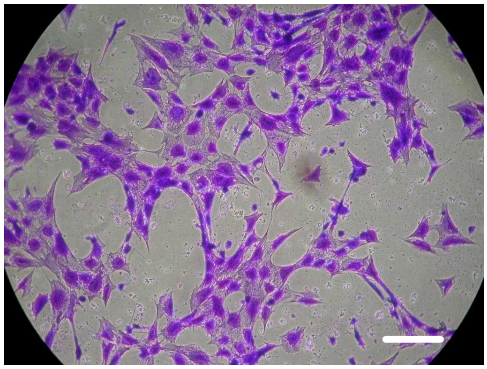
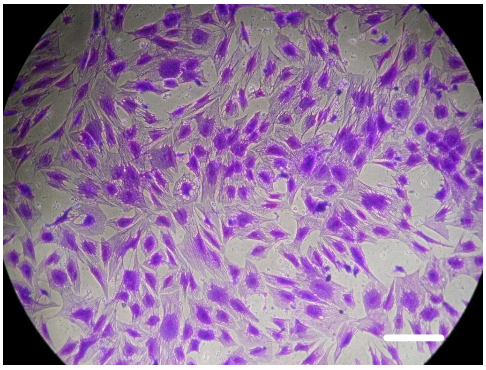


c.4 VN/IGFBP-3/IGF-I



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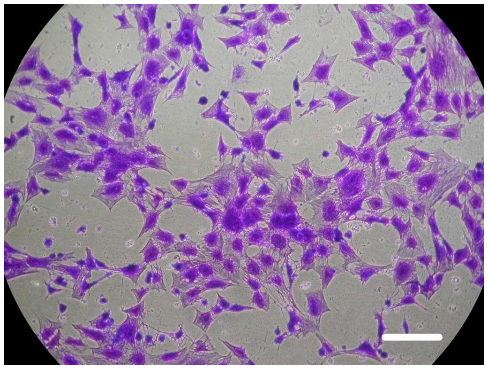
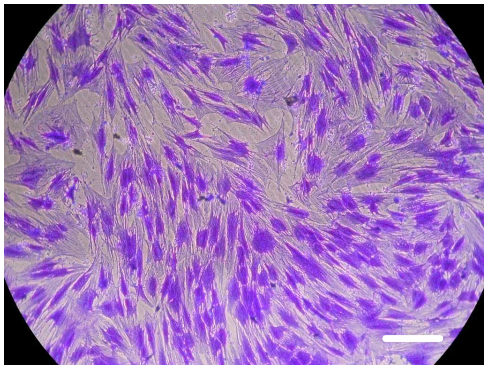
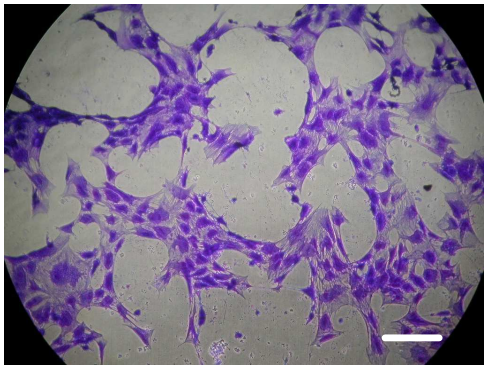


c.6 VN/IGFBP-5/IGF-I



c.7 10% FCS

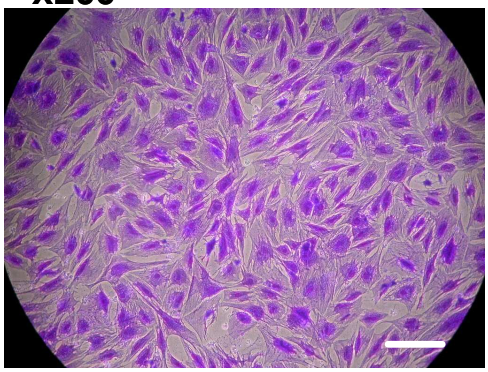


c.8 VN/TGF-$ß_1$

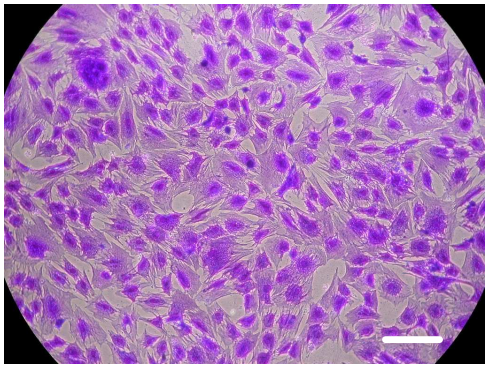
Figure 4.8c

Figure 4.9. Effect of VN concentration on IGF-I and TGF-β_1 mediated hMSC culture aggregation. Sub-confluent cultures of hMSCs were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of 11.9 nM VN (174 ng/well) and 11.9 nM IGFBP-3 or -5 (70 ng/well) and 11.9 nM IGF-I (17.4 ng/well) or 1.43 nM TGF-β_1 (7 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 72 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and digital images of each well captured at X100 magnification. Bar = 200µm.

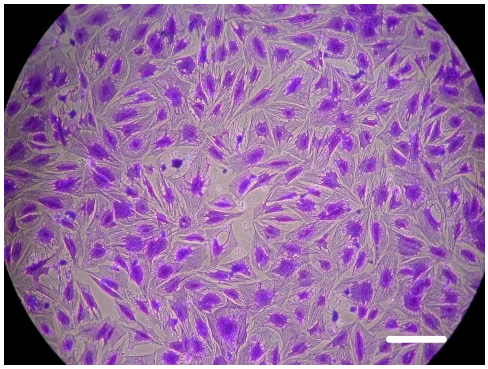
x100



a.1 VN



a.2 VN/IGF-I



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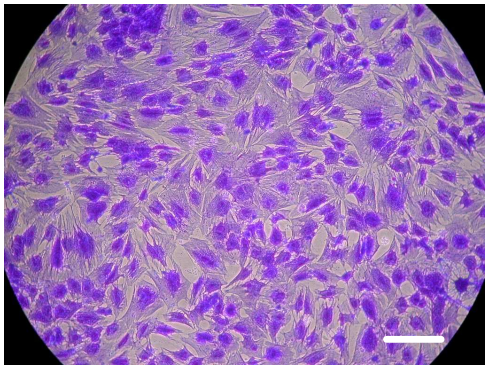
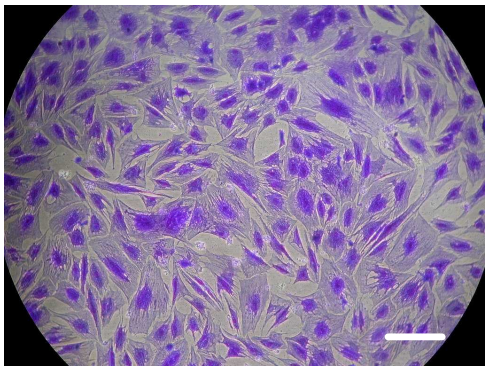
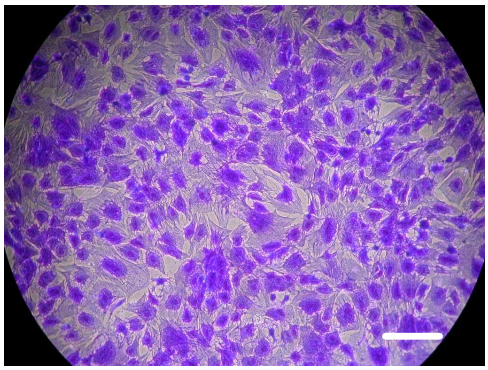


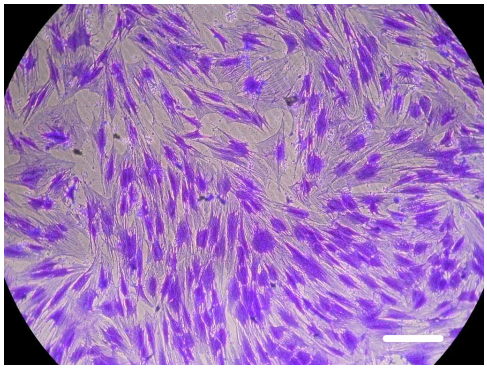
a.4 VN/IGFBP-3/IGF-I



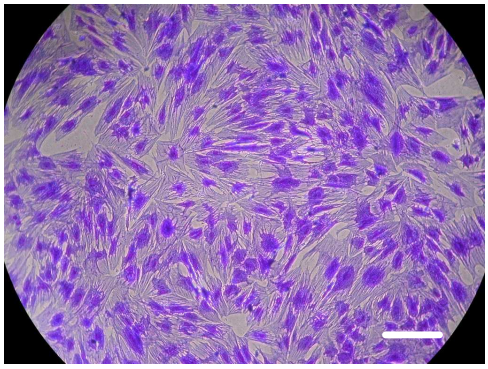
a.5 VN/IGFBP-5



a.6 VN/IGFBP-5/IGF-I



a.7 10% FCS



a.8 VN/TGF-$ß_1$

Figure 4.9

Figure 4.10. Donor effect on IGF-I and TGF-β_1 mediated hMSC culture aggregation. Sub-confluent cultures of hMSCs from 3 separate donors were harvested by trypsin digestion prior to seeding into wells of 96 well plates containing various combinations of either 3.55 nM (52 ng/well) or 11.9 nM (174 ng/well) VN and 11.9 nM IGFBP-5 (70 ng/well) and 11.9 nM IGF-I (17.4 ng/well) or 1.43 nM TGF-β_1 (7 ng/well) at a density of 5000 cells/well. Cells seeded into wells without VN or with normal growth media containing 10% FCS were employed as controls. Plates were incubated for 72 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Cells and protein were fixed with 4% paraformaldehyde and then stained with freshly prepared crystal violet. Plates were then washed, allowed to air dry and digital images of each well captured at X100 magnification. Bar = 200µm.

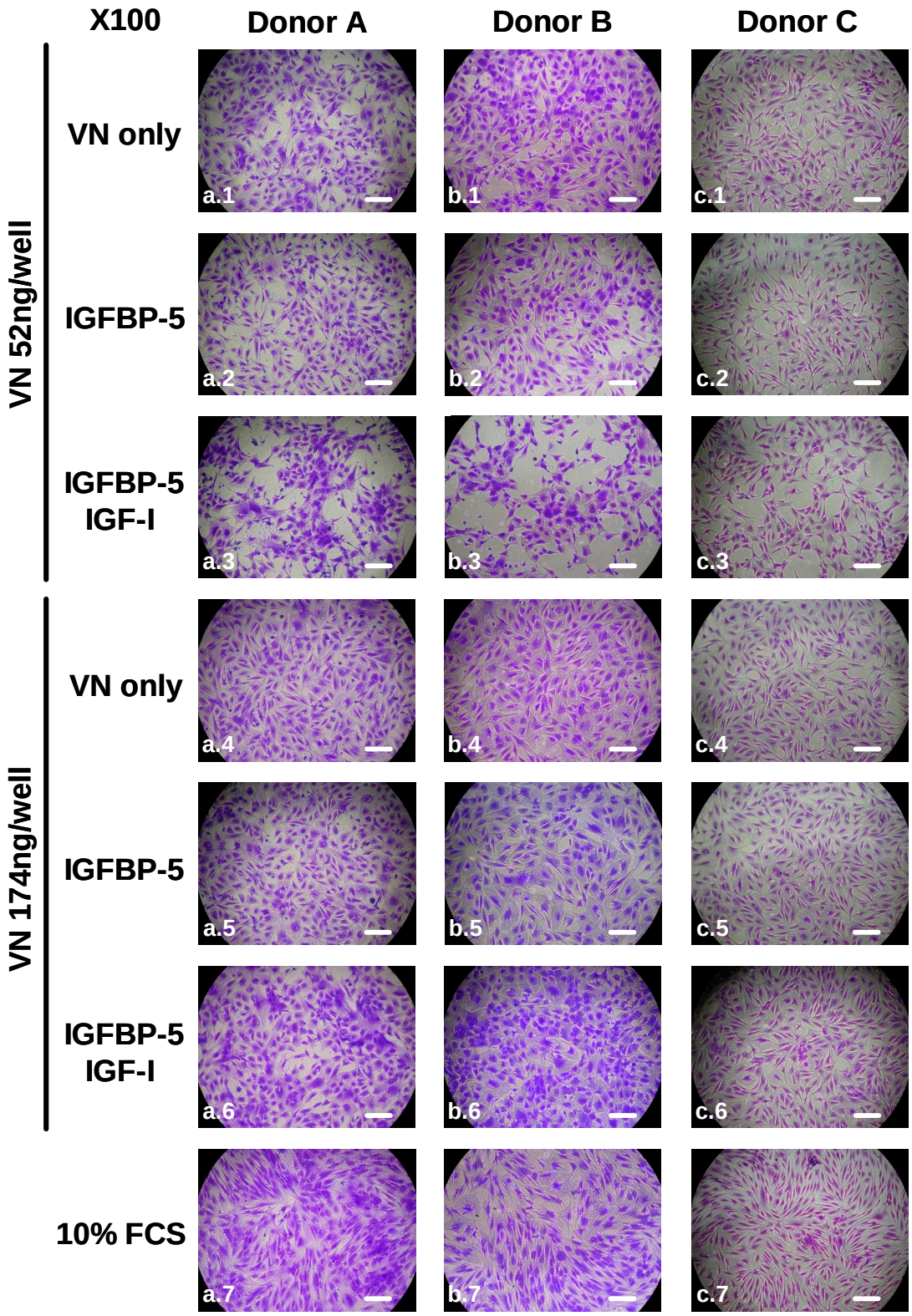

X100
Donor A
Donor B
Donor C
VN 52ng/well
VN only
IGFBP-5
IGFBP-5
IGF-I
VN 174ng/well
VN only
IGFBP-5
IGFBP-5
IGF-I
10% FCS
a.1
b.1
c.1
a.2
b.2
c.2
a.3
b.3
c.3
a.4
b.4
c.4
a.5
b.5
c.5
a.6
b.6
c.6
a.7
b.7
c.7

Figure 4.10

4.3.11 Effect of solution phase VN, IGFBP-3 or -5 and IGF-I or TGF-β_1 on expression of MMP-2 and MMP-9 by hMSCs.

Because mesenchymal cells are known to express a variety of proteases including members of the matrix metalloprotease (MMP) family of proteins (Sternlicht and Werb 2001) and because IGF-I and TGF-β_1 are both known to regulate expression of MMPs in cells of mesenchymal origin (Blumenfeld *et al.* 2002b), I hypothesised that the aggregation and contraction of cultures observed in the presence of either IGF-I or TGF-β_1 was due to the action of secreted MMPs. I therefore decided to investigate whether the presence of IGF-I or TGF-β_1 influenced the secretion of MMP-2 or -9 (members of the metalloprotease family of proteases whose substrates include IGFBP-3, -5 and VN) by gelatin zymography. Somewhat surprisingly, I found that there was no difference in MMP-2 or -9 expression in any of the treatments and that both MMP-2 and MMP-9 were expressed in their pro-form. Thus, IGF-I and TGF-β_1 did not influence the secretion of these 2 proteases into the conditioned media, nor their activation. Indeed, the data obtained for the 72 hr –VN control (hMSCs cultured in serum free media alone) suggests that hMSCs constitutively secrete MMP-2 and -9 (Fig 4.11a.1 & b.1). Each donor's cells produced similar results as demonstrated by comparison of gelatin zymograms of hMSC conditioned media derived from 2 separate donors depicted in Fig 4.11. Furthermore, MMP-2 and MMP-9 were both apparent after 24 hr and appeared to accumulate in the conditioned media over time, demonstrated by the increased lytic activity in the 48 hr and 72 hr zymograms (Fig 4.12a, b & c). Thus, hMSCs constitutively express pro-MMP-2 and pro-MMP-9 into the media. However, given that there were no discernable differences in the pro-MMP-2 or pro-MMP-9 expression between treatments, it is unlikely that either of these proteases were directly responsible for the aggregation or contraction of hMSC cultures in response to IGF-I or TGF-β_1.

Figure 4.11. Effect of solution phase VN, IGFBP-3 or -5 and IGF-I or TGF-β_1 on expression of MMP-2 and MMP-9 by hMSCs.
Conditioned media was sampled from representative wells of 72 hr cultures from separate donors prior to fixation of cells for crystal violet staining during the total protein assays as described in the materials and methods and section 4.2.6. Protease species present in conditioned media were detected and characterised with gelatin zymography as described in the materials and methods and section 4.2.8. Samples (20 µL) were separated on 10% gelatin SDS-PAGE gels. The gels were then washed in 2.5% Triton-X100 and then incubated for 48 hr at 37°C in activation buffer. Zymograms were then stained for 2 hr with Coomasie blue R250 stain de-stained in 10% acetic acid and 40% methanol (vol / vol). Gelatinase activity was then visualised as clear bands and discriminated by comparison to purified pro-MMP-2 and MMP-9 standards. IGFBP-3 or -5 are abbreviated to BP-3 or -5 for simplicity.

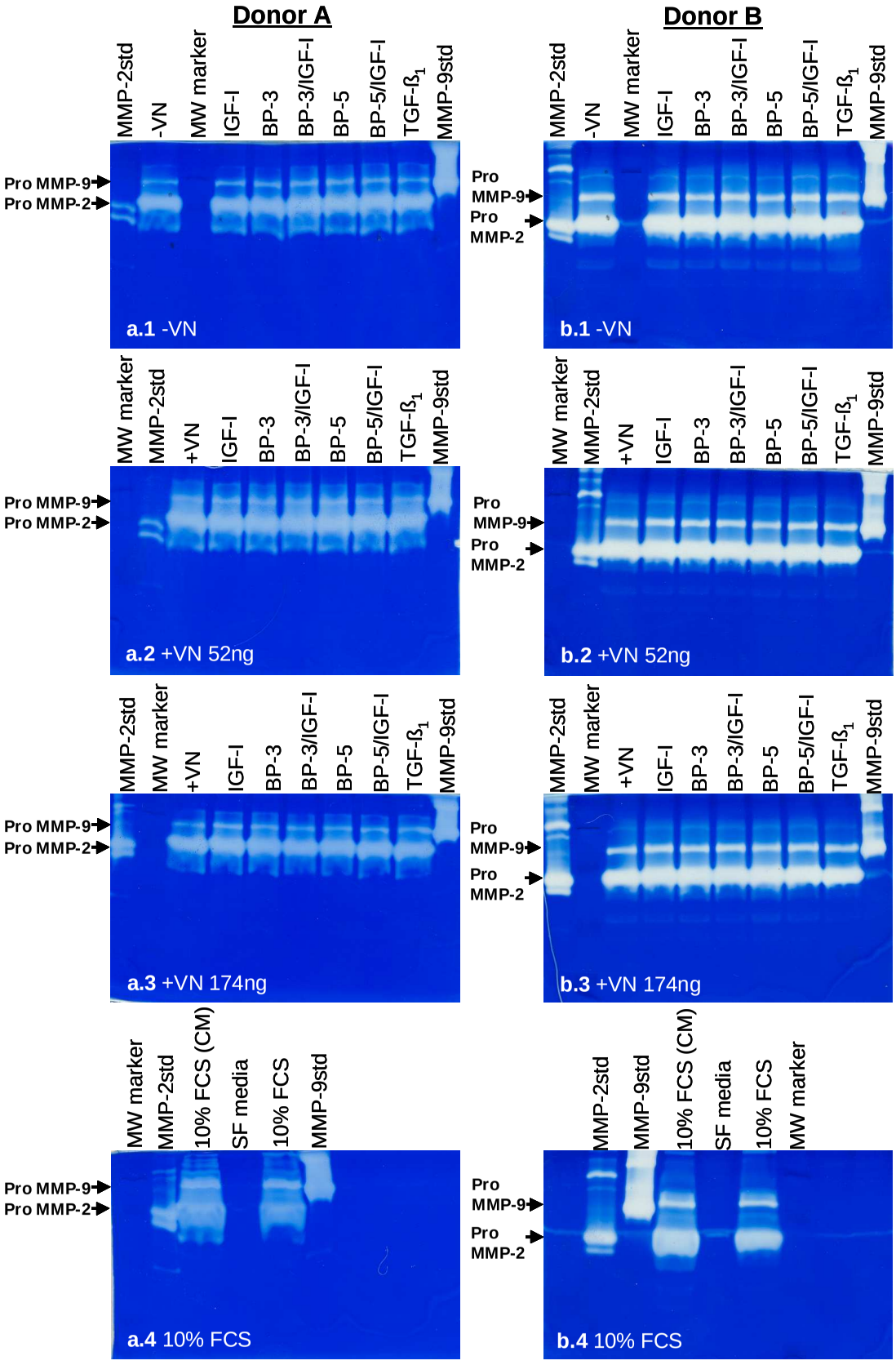

Donor A
Donor B
MMP-2std
-VN
MW marker
IGF-I
BP-3
BP-3/IGF-I
BP-5
BP-5/IGF-I
TGF-β1
MMP-9std
Pro MMP-9
Pro MMP-2
a.1 -VN
b.1 -VN
+VN
a.2 +VN 52ng
b.2 +VN 52ng
a.3 +VN 174ng
b.3 +VN 174ng
10% FCS (CM)
SF media
10% FCS
a.4 10% FCS
b.4 10% FCS

Figure 4.11

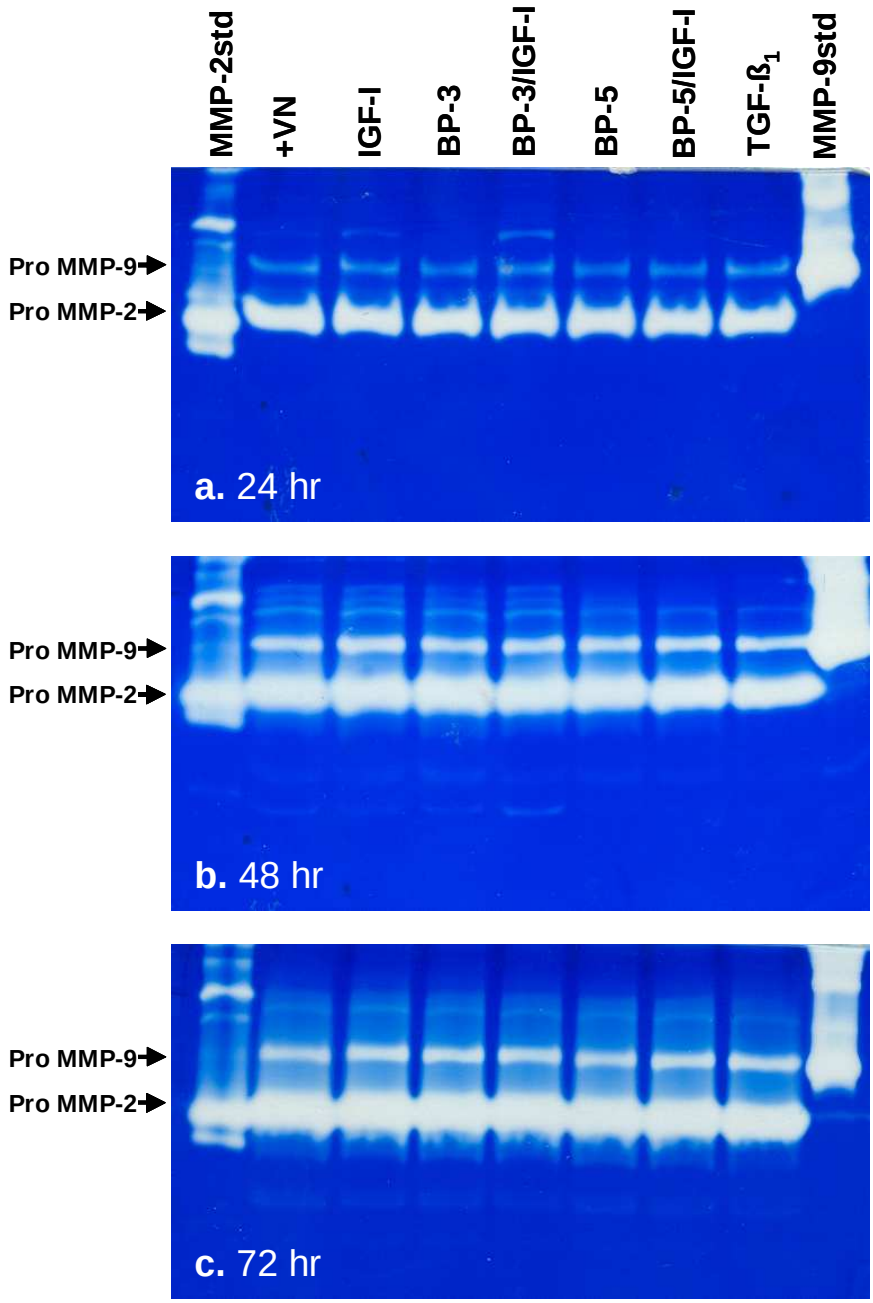


Figure 4.12. Time dependent expression of MMP-2 and –9
Conditioned media was sampled from representative wells of a) 24, b) 48 and c) 72 hr cultures from separate donors prior to fixation of cells for crystal violet staining during the total protein assays as described in the materials and methods and section 4.2.6. Protease species present in conditioned media were detected and characterised with gelatin zymography as described in the materials and methods and section 4.2.8. Samples (20 µL) were separated on 10% gelatin SDS-PAGE gels. The gels were then washed in 2.5% Triton-X100 and then incubated for 48 hr at 37 °C in activation buffer. Zymograms were then stained for 2 hr with Coomasie blue R250 stain de-stained in 10% acetic acid and 40% methanol (vol / vol). Gelatinase activity was then visualised as clear bands and discriminated by comparison to purified pro-MMP-2 and MMP-9 standards. IGFBP-3 or -5 are abbreviated to BP-3 or -5 for simplicity.

4.3.12 Degradation of IGFBP-5 by hMSC conditioned media.

The IGFBPs, IGFBP-3 and -5 are known to be susceptible to proteolytic degradation by a wide variety of proteases including MMP-2 and -9 (Fowlkes *et al.* 1995). Therefore to determine if IGFBP-5 could be potentially degraded by the conditioned media I examined hMSC-conditioned media, which had been spiked with IGFBP-5, for degradation products by western blot analysis. I found that IGFBP-5 could be degraded by conditioned media from hMSCs cultured in serum free media for 72 hr (Fig 4.13). This indicates that some entity within the hMSC conditioned media is capable of degrading IGFBP-5 and the expression of this entity is independent of any additional treatment with VN, IGFBP-3 or -5, IGF-I, TGF-β_1 or 10% FCS.

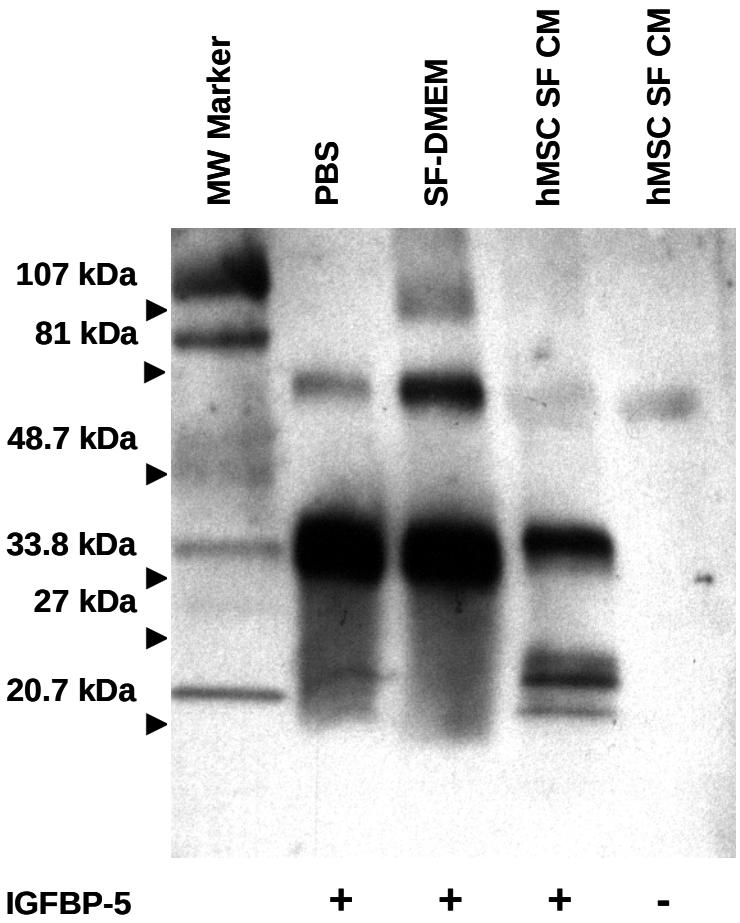


Figure 4.13 IGFBP-5 degradation by hMSC conditioned media.
Samples of conditioned media from hMSCs cultured in sf-DMEM alone, collected from the total protein assays, were incubated over night at 37°C with or without recombinant human IGFBP-5 and then run under reducing conditions on a 4%-20% gradient SDS-PAGE gel. Recombinant human IGFBP-5 in phosphate buffered saline or sf-DMEM were employed as controls. Proteins were then transferred to a nitrocellulose membrane prior to interrogation with a chicken anti IGFBP-5 polyclonal antibody and subsequent 1° antibody detection with a rabbit anti chicken-HRP conjugated 2° antibody and chemiluminescent substrate. Chemiluminescence was then captured on photographic film.

4.4 DISCUSSION

There has been a vast body of literature generated in the last decade describing the development and efficacy of hMSCs as a reparative therapeutic, whether as part of a tissue engineered biomaterial construct for bone repair or re-infusion of *ex vivo* expanded cells for treatment of cardiac failure (Bruder *et al.* 1998a; Bruder *et al.* 1998c; Young *et al.* 1998; Koc *et al.* 1999; Ohgushi and Caplan 1999; Oreffo and Triffitt 1999; Richards *et al.* 1999; Koc *et al.* 2000; Bianco *et al.* 2001; Caplan and Bruder 2001; Minguell *et al.* 2001; Derubeis and Cancedda 2004; Hermann *et al.* 2004). However, *in vitro* culture of these cells is still largely dependent on the use of animal-derived products, particularly FCS, which remains essentially undefined. With the changing regulatory climate concerning tissues for therapeutic applications, the development of culture methodologies for hMSCs which include defined media components and do not include poorly defined animal products has become a vital area of research within the field of tissue engineering (Kujawa *et al.* 1989; Rousche *et al.* 2001; Fukumoto *et al.* 2003; Stute *et al.* 2004; Meuleman *et al.* 2006). The concept of serum free media development was consistent with the investigations into the effects of pre-bound combinations of the ECM protein VN and the IGFs on cell function, including osteoblasts as discussed in the previous chapter.

With this chapter I report broad preliminary studies into the effect of the ECM glycoprotein VN in association with various combinations of IGFBP-3 or -5, IGF-I or TGF-β_1, on hMSC behaviour in a serum-free environment. Initially, I attempted to initiate hMSC culture from crude bone marrow-derived mononuclear cell fractions on pre-bound combinations of VN and IGFBP-3 or -5 and IGF-I, or VN and TGF-β_1, by performing colony forming unit fibroblastic assays (Figure 4.1). Over 7 days of culture few cells had appeared to attach to the coated culture surfaces (Figure 4.1a & b) whereas, colony formation and fibroblastic cells were clearly visible in cultures exposed to 10% FCS, as expected (Figure 4.1c & d). Thus the donor cells used in these assays were viable and capable of forming healthy colonies and continued to develop beyond 7 days of culture. Based on this initial result I decided to pre-bind the VN but to leave the unbound fraction and growth factors in solution for subsequent assays in order to try and: 1) initiate fibroblastic cell attachment; and 2)

prolong the viability of any attached cells by leaving the unbound fractions of the VN and the growth factors in solution.

Initially, this strategy seemed successful as after 1 day of culture a number of fibroblast-like cells were attached to the culture surface, although unexpectedly these were not incorporated into colonies but were discrete and seemingly randomly arranged throughout the wells (Figure 4.1e & g). After 3 days of culture the number of attached fibroblastic cells had increased, however, it is not clear whether the observed increase was due to cell proliferation or due to increased cell attachment from the existing population of mononuclear cells (Figure 4.1f & h). Curiously, the cells attached to the protein coated surface appeared more spindle shaped and smaller than the fibroblast-like cells present in the 10% FCS. Furthermore, the number of attached cells did not continue to increase but rather declined over the next few days of culture until few cells remained attached (data not shown). These data suggest that VN together with either IGFBP-3 or 5 and IGF-I or TGF-β_1 can initiate fibroblast formation from crude preparations of bone marrow mononuclear cells but cannot sustain them past about 3-4 days of culture. Moreover, pre-bound VN in combination with pre-bound IGFBPs and IGF-I could not initiate fibroblast attachment from the bone marrow preparations.

It is possible that non mesenchymal cell types present in the crude mix of mononuclear cells depleted the VN and growth factors, whereas, when the components were present at a higher concentration there may have been sufficient VN and growth factor in the media to facilitate fibroblast formation. It also remains unclear as to whether these cells were indeed hMSCs. A possible means of testing the hypothesis that these cells were in fact hMSCs would be to remove all non bound cells at the earliest convenience and replace the media with 10% FCS to ascertain how many of the discrete cells would go onto form colonies which could then be assayed for their multilineage potential.

To the best of this author's knowledge this is the only study reported where the adherent cells, derived from the mononuclear cell fraction of bone marrow, attached discreetly rather than forming in colonies. Gronthos *et al,* (2001) has previously reported that STRO-1^+ hMSCs plated on a variety of ECM glycoproteins including

VN and FN formed equivalent numbers of colonies compared to 20% FCS. It is important to note, however, that the growth media was supplemented with PDGF, EGF, bovine insulin, BSA, human low density lipoprotein, iron-saturated human transferrin, L-glutamine, dexamethasone sodium phosphate, L-ascorbic acid 2-phosphate and β-mercaptoethanol and the cells were sourced from healthy human donors (Gronthos *et al.* 2001), unlike the present study which utilised cells sourced from elderly patients presenting for total joint replacement surgery. Taken together, the differences in methodology between the study performed by Gronthos and co-workers and the current study, may account for the difference in results obtained.

Due to these results I then decided to measure the functional responses of hMSCs which had been previously expanded *in vitro*, to various combinations of VN, IGFBP-3 or -5, IGF-I or TGF-β_1, where the unbound fraction of VN and the growth factors remained in solution. Firstly, I measured the metabolic activity of hMSCs exposed to the various combinations of VN and growth factors. Observation of these cultures revealed that cultured hMSCs were able to attach and spread on VN alone, which is consistent with previous studies which demonstrated that hMSCs express a range of integrins including the VN receptor, $\alpha_v\beta_3$ integrins (Conget and Minguell 1999; Gronthos *et al.* 2001). Furthermore, Salasznyk and co–workers (2004) recently performed hMSC adhesion assays on a range of pre-bound purified ECM proteins and found that hMSCs adhered equally well to collagens type I and IV, FN and VN, although these assays were performed in the presence of FCS and thus other factors present in the serum will have influenced these findings (Salasznyk *et al.* 2004b).

The major influence on hMSC metabolic activity in the present study was the presence or absence of VN, indicating that cell adhesion was vital in this system in order for the hMSCs to respond to any of the added growth factors. Furthermore, the addition of IGF-I alone with VN slightly enhanced the response of hMSCs while the addition of either of the IGFBPs alone with VN had no effect above that of VN alone. Moreover, the addition of IGF-I together with either of the IGFBPs and VN did not enhance the response of hMSCs compared to that of IGF-I alone with VN. In addition, I found that increasing the concentration of VN did not increase the metabolic activity of hMSCs after 72 hr in response to IGF-I. These data are similar

to the findings of previous studies with hMSCs which found no increase in hMSC number when treated with either IGF-I, IGFBP-3 or IGF-I and IGFBP-3 together, although these responses were significantly enhanced over the control treatment alone (Kveiborg *et al.* 2001a) in contrast to the responses observed herein which were similar to the +VN control. However, in the study by Kveiborg *et al*, (2001a) the authors first cultured the cells in the presence of 10% FCS which was removed following 48 hr of culture and replaced with media containing 0.5% FCS supplemented with either IGF-I, IGFBP-3 or both; thus the elevated response compared to the control (0.5%FCS in media only) may have been due in part to additional factors present in the FCS (other than VN) which were not present in my study.

Between 24 hr and 48 hr hMSC metabolic activity declined in all treatments, including the 10% FCS control, then stabilised or increased between 48 hr and 72 hr depending on the treatment (Fig 4.2, 4.3 and 4.4). In regards to the treatments without VN, the decline in metabolic activity may have been due to a reduction in cell number and / or reduced mitochondrial enzyme activity. An increase in metabolic activity may similarly be due to an increase in cell number or mitochondrial enzymatic activity. Bearing these limitations in mind, only TGF-β_1 together with VN at either 52 ng/well or 174 ng/well were able to stimulate metabolic activity after 72 hr that was significantly higher than the 10% FCS control ($p<0.05$ or $p<0.01$ respectively)(Fig 4.4a). This observation suggests that TGF-β_1 has a direct stimulatory effect on hMSC metabolic activity, via its down stream signalling effectors, or indirectly by stimulating synthesis and expression of IGFBP-3 and IGF-I as described previously (Kveiborg *et al.* 2001b).

The possibility that the enhanced metabolic activity measured for hMSCs exposed to TGF-β_1 is a response to endogenously produced IGFBP-3 and IGF-I is intriguing because the response of hMSCs exposed to the exogenously added IGFBP-3 and IGF-I was equivalent to 10% FCS while the response to TGF-β_1 at either concentration of VN (52 ng/well or 174ng/well) were significantly higher than the response to 10% FCS ($p<0.05$ or $p<0.01$ respectively) (Fig 4.3 and 4.4). However, the exogenous IGFBP-3 used in this study was a mutated isoform (N109D) produced in *E.coli* and is non-glycosylated (~35 kDa) (Upstate, Waltham, MA, USA) whereas

hMSCs exposed to TGF-β_1 produce 38 kDa and 42 kDa isoforms of IGFBP-3 which likely represent partially and fully glycosylated isoforms (Kveiborg *et al.* 2001b; Kveiborg *et al.* 2002). Thus the basis of the difference in response remains unclear, although, glycosylation status of IGFBP-3 or the level of expression of either IGFBP-3 or IGF-I may play a role in the level of response.

Human lung fibroblasts have also been shown to increase BrdU incorporation in response to VN and TGF-β_1 compared to TGF-β_1 alone and this response was due to direct interaction of $\alpha_v\beta_3$ integrins and the TGF-β type II receptor (TGFβIIR)(Scaffidi *et al.* 2004). Specifically, TGF-β_1 increased the cell surface expression of $\alpha_v\beta_3$ integrins, co-localisation and immunocomplex formation between the TGFβIIR and $\alpha_v\beta_3$ integrin, and up regulated cyclin D1 to result in a synergistically enhanced proliferation response to ligand occupancy of the $\alpha_v\beta_3$ integrin and the TGFβIIR (Scaffidi *et al.* 2004). Taken together, these data support my finding that hMSC metabolic activity was synergistically enhanced after 72 hr in response to TGF-β_1 and VN at 52 ng/well and was further enhanced by VN at 174ng/well. Furthermore, these findings suggest that the observed increase in synergistic responses may be due to the combined effects of: 1) an increase in integrin binding sites and a concomitant increase in cell surface $\alpha_v\beta_3$ integrins (ie an ability to respond to the increase in VN) and 2) perhaps an autocrine/paracrine response to endogenously expressed IGFBP-3 and IGF-I. However, further investigation into the specific mechanisms underpinning this response is necessary in order to draw specific conclusions about the role of TGF-β_1, VN, IGFBP-3 or IGF-I and their respective receptors in the observed responses.

While measurement of metabolic activity can provide an indication of a culture's response to a given treatment in real time it does not necessarily give an indication of the cumulative response to a given treatment. To this end I decided to measure the change in hMSC derived total protein over 72 hr by staining cultures with the cationic dye crystal violet. In contrast to the observed metabolic activity response, there was no decline in hMSC total protein, thus indicating that the observed decline in metabolic activity after 24 hr was probably not due to a decrease in cell number but is more likely due to a decrease in mitochondrial enzyme activity. A decrease in

cell number would be likely to correspond to a decrease in total protein levels (Figs 4.5, 4.6 & 4.7). Again I found that the major contributing factor to increasing hMSC total protein in this system was the presence of VN. Although, in contrast to the metabolic activity response, the combination of VN (especially at 174 ng/well) together with either of the IGFBPs and IGF-I seemed to slightly enhance the total protein accumulation after 72 hr (Figs 4.5a & 4.6a) compared to VN alone or IGF-I in the presence of VN, although this increase was not significant. In addition, the increase in total protein between 48 hr and 72 hr for hMSCs exposed to IGFBP-3 or -5 and IGF-I in the presence of VN at 174 ng/well was equivalent to that of hMSCs exposed to 10% FCS.

Jia and Heersche (2000) reported that IGF-I could stimulate osteoprogenitor cell proliferation. However, others have reported that IGF-I has no effect on hMSC proliferation or differentiation (Jia and Heersche 2000; Walsh *et al.* 2003). Andress and Birnbaum (1992) have previously shown that an osteoblast derived isoform of IGFBP-5 enhances IGF-I stimulated osteoblast mitogenesis while purified IGFBP-3 dose dependently inhibited IGF-I stimulated osteoblast mitogenesis (Andress and Birnbaum 1992). The authors postulated that the inhibitory action of IGFBP-3 was due to sequestration of IGF-I by IGFBP-3 in solution phase, thereby inhibiting IGF-I association with the IGFIR. Of particular interest, IGF-I in the presence of the bone matrix protein, osteopontin, a ligand for $\alpha_v\beta_3$ integrin, was shown to stimulate porcine smooth muscle cell (pSMC) proliferation and was further enhanced to levels similar to that observed for 10% FCS by the addition of IGFBP-5 (Nam *et al.* 2002), thus corresponding with my findings in hMSCs. Further, the interaction between IGFBP-5 and VN has been shown to be critical for the enhancement of IGF-I mediated pSMC DNA synthesis (Nam *et al.* 2002). Indeed research in our laboratory has confirmed and expanded the concept of enhanced cellular response to complexes composed of select IGFBPs and IGF-I, including IGFBP-3 and -5, bound to VN (Kricker *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005). These findings are supported by the results detailed in the previous chapter and the present study adds to the growing body of experimental evidence.

The measurement of total protein in hMSC cultures exposed to TGF-β_1, together with VN at either 52 ng/well or 174 ng/well, do not reflect the results obtained from the metabolic activity assays in that the large increase in metabolic activity between 48 hr and 72 hr was not accompanied by a similar increase in protein accumulation in the same time period (Fig 4.7a & b). It is possible that the high metabolic activity stimulated in hMSCs exposed to 174 ng/well VN and TGF-β_1 may result in increased protein expression at some time after 72 hr and therefore would not be evident in the total protein measurement at this time. In order to test this hypothesis, a more complete study of the functional response of hMSCs to VN and TGF-β_1, with measurements taken over a longer period, is required to more accurately track the changes in response to these 2 factors. Such a study might also incorporate an analysis of the expression of $\alpha_v\beta_3$ integrins, IGFBP-3, IGF-I and the IGFIR. Coincidental interrogation of the signalling pathways of these receptors will assist a fuller understanding of the mechanisms underlying the responses observed in this study.

Interestingly, my morphological assessment revealed that hMSC cultures exposed to VN at 52ng/well in the presence of either IGF-I or TGF-β_1 underwent contraction and / or aggregation in a time-dependent manner (Figs 4.8a, b & c). In the first instance this aggregation appeared to be a loss of cells from the culture, possibly through loss of cell adhesion or cell death. However, closer analysis revealed that despite the phenomenon occurring in the cultures exposed to IGF-I or TGF-β_1, most obvious after 72 hr (Fig 4.8c.2, 4, 6 & 8), the total protein data suggested there was little or no change in total protein content between any of these treatments and the total protein content of hMSCs exposed to VN at 52 ng/well alone. This suggests there was not a significant decrease in cell number during this time. Furthermore, cultures which were exposed to either IGF-I or TGF-β_1 in the presence of VN at 174 ng/well appeared to be protected from this effect (Fig 4.9). While few studies have previously described this phenomena, Hurley *et al*, (1994) described the aggregation of mouse osteoblastic cells exposed to TGF-β_1 (Hurley *et al.* 1994) and more recently Karsdal and co-workers (2001) found that MC3T3-E1 cells cultured in serum free media and exposed to TGF-β_1 developed identical culture morphologies as I have described here for the hMSCs. In addition when the MC3T3-E1 cells were

cultured on a type I collagen substratum (tissue culture plates pre-coated with 30 µg/mL of type I collagen) under similar conditions, the effect was greatly diminished (Karsdal *et al.* 2001). Of interest, focal adhesion kinase (FAK) expression was increased and phosphorylation / activation of ERK1/2 and p38 MAP kinases, but not Jun N-terminal Kinase (JNK) was also increased in response to TGF-β_1 which may partly explain the increase in metabolic activity in hMSCs measured in my own study.

Signalling pathway specific inhibitors in the Karsdal *et al* studies revealed that the p38 MAP kinase pathway but not the ERK1/2 or JNK pathways, were involved in the morphological response to TGF-β_1 (Karsdal *et al.* 2001). This phenomenon may be further explained, in part, by the fact that TGF-β_1 is also known to induce plasminogen activator inhibitor (PAI-1) expression (Boehm *et al.* 1999; Kutz *et al.* 2001). PAI-1 has recently been shown to mediate cell detachment from VN substrata by initiating endocytosis of uPA-uPAR-α_v integrin complexes via complex formation with the low density lipoprotein (LDL) receptor related protein (LRP) (Czekay *et al.* 2003). These observations sit well with my observations that the aggregation response was attenuated in the presence of higher VN concentration, a condition in which more integrin binding sites are occupied. This is further supported by the finding that by over expressing the number of $\alpha_v\beta_3$ integrins on the surface of CHO cells, PAI-I mediated cell detachment is drastically reduced (Czekay *et al.* 2003). The present study is the first to: 1) describe this phenomenon in hMSCs; and 2) demonstrate that by increasing the concentration of VN the observed response can be attenuated. While a similar response has been reported for osteoblasts exposed to parathyroid hormone, where they adopted a stellate morphology (Tram *et al.* 1993; Karsdal *et al.* 2001), I am unaware of any studies, other than the present study, which reports a similar response to IGF-I.

The molecular mechanism mediating the IGF-I mediated response remains to be elucidated. I initially thought that the observed growth factor mediated aggregation effect may be due to secretion of proteases which in turn affect hMSC attachment to the substratum. In addition, IGFBP-3 and -5 are known substrates for a number of proteases secreted by mesenchymal cells, including osteoblasts and of these MMP-2

features predominantly (Fowlkes *et al.* 1995; Sternlicht and Werb 2001). Indeed I found that purified IGFBP-5 could be degraded by factors within hMSC conditioned media (Fig 4.13). However, I found that there was no correlation between the functional expression of either MMP-2 or -9, as determined by gelatin zymography, and the growth factor-induced hMSC culture phenotype. I observed that both MMP-2 and MMP-9 were present in the hMSC conditioned media in their pro-form, indicating that neither IGF-I or TGF-β_1, nor indeed any other treatment, had an effect on MMP-2 or -9 activation (Fig 4.11). Indeed, MMP-2 and -9 accumulate in the conditioned media over time (Fig 4.12). Similar results have been reported for MC3T3-E1 cells exposed to TGF-β_1, where TGF-β_1 was found to not affect the expression of MMP-2, yet induced MMP-13 expression (Karsdal *et al.* 2001). However, Karsdel *et al* did not clarify whether the secreted MMP-2 was the active or in-active pro-form.

Given that hMSCs exposed to no treatment whatsoever (the –VN control) also expressed pro-MMP-2 and -9 I concluded that both of these proteases are constitutively expressed by hMSCs (Lane 2, Fig 11a.1 & b.1). It appears this is relatively common for a number of different cell types, especially for MMP-2 (Sternlicht and Werb 2001). Interestingly, VN has been shown to induce MMP-2 expression in melanoma cells (Bafetti *et al.* 1998) and both MMP-2 and -9 interact or cooperate with $\alpha_v\beta_3$ integrins to regulate cell migration and invasion (Brooks *et al.* 1996; Rolli *et al.* 2003). Others have further reported that over expression of TIMP-2, an MMP-2 inhibitor, resulted in reduced MMP-2 activity (as expected) and restored attachment and spreading of melanoma cells on a VN substratum in spite of also demonstrating that activated MMP-2 did not degrade the VN substrate (Ray and Stetler-Stevenson 1995). Indeed solution phase VN has been shown to not be susceptible to MMP-2 mediated proteolysis (Bafetti *et al.* 1998). I did not investigate the expression of TIMPs by hMSCs in this study. Further investigations of the fate of the proteins used in this study are required to more fully understand the dynamics of biologically active species within such serum free culture environments.

In conclusion, the data presented in this chapter indicate that VN is a valuable matrix component that provides a significant initial stimulus for the serum-free culture of

hMSCs and that in the presence of other factors, such as TGF-β_1, additional stimuli can be achieved. In addition the concentration of VN is an important factor in the maintenance of culture phenotype and integrity and further investigation into the effect of endogenously expressed proteases on the functionality of matrix components, growth factors or other constituents is warranted. Lastly, a detailed investigation of the mechanisms of TGF-β_1 action on hMSC function in the serum-free environment is required.

CHAPTER 5:

MULTI-PROTEIN COMPLEXES DETERMINE SaOS-2 CELL FUNCTIONAL RESPONSES

5.1 INTRODUCTION

Fibronectin (FN) is a 400-500 kDa, multifunctional plasma and ECM glycoprotein which typically exists as a homodimer of two 200-250 kDa disulfide linked subunits. The molecular weight (MW) of FN can vary depending on alternative splice variations (Tamkun *et al.* 1984), although FN derived from plasma commonly has a lower MW (~450 kDa) than ECM - derived FN (~500 kDa). Plasma FN is largely synthesised by hepatocytes in the liver and is present in plasma at ~300 µg/mL and is soluble at physiological pH. In contrast, ECM FN is not soluble at physiological pH (Yamada and Kennedy 1979). FN is also a component of mature bone ECM and has been shown to mediate a number of cellular functions *in vitro* including cell attachment, spreading, migration, proliferation and differentiation (Grzesik and Robey 1994; Degasne 1999; Garcia *et al.* 1999; Xu *et al.* 2004). These functional effects are dependent on the type of cells under investigation, the particular suite of integrins which they express and the substrate-mediated conformation of the FN molecules themselves (Garcia *et al.* 1999; Danen *et al.* 2002). Cell attachment to FN is mediated by a wide range of different integrins and significant among these are the RGD binding integrins $\alpha_5\beta_1$ and $\alpha_v\beta_3$ (Ruoslahti and Pierschbacher 1987; Ruoslahti 1988; Plow *et al.* 2000; Pankov and Yamada 2002). In addition to the RGD recognition sequence, FN has a number of other integrin binding sites which facilitate the binding of $\alpha_2\beta_1$, $\alpha_4\beta_1$ and $\alpha_4\beta_7$ integrins (Plow *et al.* 2000). Furthermore, cell surface integrins, in particular the $\alpha_5\beta_1$ integrin, mediate FN fibril assembly into the ECM via the ability of this integrin to support high levels of RhoA-GTPase activity (Danen *et al.* 2002). RhoA-GTPase is involved in regulation of cell-cell adhesion, as well as actin stress fibre and focal contact formation (Rottner *et al.* 1999).

FN is capable of binding to a number of ECM proteins including, most notably, type I collagen (Col-I), especially in its denatured form, gelatin (Engvall and Ruoslahti 1977; Ruoslahti 1988; Pankov and Yamada 2002). Other ECM components which are known to interact with FN include type IV collagen, fibrin, heparin and heparan / chondroitin / dermatan sulphate proteoglycans (Ruoslahti 1988; Romberger 1997). More recently FN has been shown to bind both IGFBP-3 and -5 (Gui and Murphy 2001). This interaction between IGFBP-5 and FN has recently been shown to

negatively modulate IGF-I dependent action on mouse embryonic cell (MEC) migration by enhancing proteolytic degradation of IGFBP-5, although the identity of the particular protease, or proteases, involved in this event remain unresolved (Xu *et al.* 2004). Additionally, FN has been shown to prevent apoptosis in mature osteoblasts and this effect was further enhanced by the addition of TGF-β_1 (Globus *et al.* 1998). The authors found that FN and TGF-β_1 cooperated to regulate the survival of mature osteoblasts *in vitro*.

Similar to VN, FN molecules also have a monolayer saturation limit when bound to tissue culture plastic which has been measured at 0.36 $\mu g/cm^2$ (Pitt *et al.* 1989; Garcia *et al.* 1999). However, interrogation of high concentrations of pre-bound FN with monoclonal antibodies revealed that multilayering of the FN molecules was possibly occurring, although it was unclear whether this effect was due to steric hindrance of antibody binding to FN molecules (Underwood *et al.* 1993).

FN shares many of the desirable properties of VN for tissue engineering applications, such as the ability to adhere to a variety of tissue culture and biomaterial surfaces, the ability to facilitate cell attachment to those surfaces (Steele *et al.* 1995; Carvalho *et al.* 1998; McFarland *et al.* 1999; Ogura *et al.* 2004; Sim *et al.* 2004; Deligianni *et al.* 2005; Lee *et al.* 2006), activation of integrin mediated signalling pathways (Carvalho *et al.* 1998; Krause *et al.* 2000) and the ability to bind IGFBP-5 (Gui and Murphy 2001; Xu *et al.* 2004). In view of this I hypothesised that pre-bound combinations of FN, IGFBP-5 and IGF-I could stimulate similar functional responses in SaOS-2 cells to those observed in the presence of VN, IGFBP-5 and IGF-I. Furthermore, VN, FN, IGFBP-5, IGF-I and TGF-β_1 have each been linked to mesenchymal stem cell, and / or osteoblast differentiation processes (Dedhar 1989; Dedhar *et al.* 1989; Moursi *et al.* 1996; Moursi *et al.* 1997; Kim *et al.* 2003; Sakaguchi *et al.* 2004; Salasznyk *et al.* 2004b), prompting me to evaluate the effect of specific combinations of these factors on osteoblast specific gene expression, namely cbfa-1, ALP and Col-1 genes.

SaOS-2 cells express both $\alpha_v\beta_3$ and $\alpha_5\beta_1$ integrins, the VN and FN receptors respectively (Koistinen *et al.* 1999; Livak and Schmittgen 2001; Postiglione *et al.*

2003; Kilpadi *et al.* 2004). They also express the IGF-I and the type I, type II and III (betaglycan) TGF-β receptors (Takeuchi *et al.* 1995; Bilbe *et al.* 1996; Nasu *et al.* 2000; Grey *et al.* 2003; MacEwen *et al.* 2004). Moreover, SaOS-2 cells, being an osteosarcoma cell line, are far easier to culture and do not require the long culture periods required for hMSCs or primary osteoblasts. Therefore, the studies in this chapter utilise SaOS-2 cells to evaluate: 1) osteoblastic responsiveness to various combinations of FN, IGFBP-5 and IGF-I or VN and FN; and 2) osteoblastic differentiation marker expression in cells exposed to select pre-bound combinations of VN, FN and growth factors, such as IGFBP-5, IGF-I, EGF and TGF-β_1.

5.2 EXPERIMENTAL PROCEDURES

Full details of both the materials and methods used in the generation of the data presented in this chapter are described in chapter 2. The following are brief summaries of the materials and experimental procedures used for the generation of data presented in section 5.3.

5.2.1 Materials

For full details of the materials used in generating the data for this chapter please refer to section 2.1. Purified human VN (Promega), IGFBP-5 (Dr Sue Firth) and IGF-I (GroPep), were obtained as detailed in the previous chapters. Other proteins used in the generation of the data for this chapter included recombinant human TGF-β_1 and recombinant human bFGF (Chemicon Pty / Ltd), purified human FN (Sigma-Aldrich), and recombinant human EGF (Invitrogen). Tissue culture reagents were as described in previous chapters and detailed in chapter 2.

Other key reagents used to generate the data presented in this chapter include Fraction V RIA grade BSA (Calbiochem), First Strand (AMV) cDNA synthesis kit (Roche Diagnostics), CyQUANT® reagent (Molecular Probes / Invitrogen), Sigmacote®, Dexamethasone, Glycerol-2-phosphate and Tri-Reagent (Sigma-Aldrich), L-Ascorbic acid 2-phosphate tri-sodium salt (Wako) and SYBR® green PCR master mix (Applied Biosystems).

5.2.2 Pre-binding of Proteins

For full details of this method please refer to sections 2.3 and 2.4. SaOS-2 cell migration was assessed on pre-bound combinations of FN, IGFBP-5 and IGF-I. Briefly, the underside of Transwell™ tissue culture plate inserts (Costar) were coated with 1 mL of 6 µg/mL FN / sf-αMEM or sf-αMEM alone for 4 hr in a humidified 5% CO_2/95% air atmosphere at 37°C followed by 2 washes with 1 mL of 0.5% BSA / HBB. Duplicate 0.5 mL aliquots of IGFBP-5 (0.1 µg/mL) in 0.05% BSA / HBB / sf-αMEM, together with either 0.5 mL IGF-I (0.025 µg/mL) in 0.05% BSA / HBB / sf-αMEM or 0.5 ml of 0.05% BSA / HBB / sf-αMEM, was added to wells with, or without, FN and incubated at 4°C overnight. Following incubation, the lower chambers were washed twice with 1 mL 0.05% BSA / HBB / sf-αMEM prior to the addition of 1 mL 0.05% BSA / HBB / sf-αMEM to the lower chamber of each Transwell™ and incubation until required.

SaOS-2 proliferation was assessed on pre-bound combinations of VN, FN, IGFBP-5 and IGF-I. Specifically, 52 µL of 1 µg/mL VN / sf-αMEM, 6 µg/mL FN / sf-αMEM or sf-αMEM alone was added to each well of a 96-well tissue culture plate and incubated for 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following removal of ±VN or FN / sf-αMEM solution, wells were washed with 0.5% BSA / HBB. Wells were then blocked with 1% BSA / HBB, incubated for 30 mins in the above conditions and each well was washed with 0.5% BSA / HBB followed by addition of 43.5 µL of IGFBP-5 (1.6 µg/mL) in 0.5% BSA / HBB either alone, or in combination with IGF-I (0.4 µg/mL), and with or without VN. The plates were incubated at 4°C overnight.

SaOS-2 cell osteoblast marker expression was assessed on the following combinations of pre-bound proteins: VN alone; VN / IGFBP-5 / IGF-I; VN / TGF-β_1; and VN / FN / EGF. Specifically, 1.5 mL of VN / sf-αMEM (1 µg/mL) or FN / sf-αMEM (6 µg/mL) or VN / FN / sf-αMEM (1 µg/mL / 6 µg/mL) were added to individual wells of a 6-well tissue culture plate and incubated for 2 hr in a humidified 5% CO_2/95% air atmosphere at 37°C. Following removal of VN / ± FN sf-αMEM solutions, wells were washed with 0.5% BSA / HBB and blocked with 1%

BSA / HBB for 30 mins in the above conditions, after which wells were washed again with 0.5% BSA / HBB. Specific wells then received 1.263 mL of IGFBP-5 / IGF-I / sf-αMEM (160 ng/mL IGFBP-5 / 40 ng/mL IGF-I), TGF-β_1 / sf-αMEM (115.2 ng/mL), EGF / sf-αMEM (115.2 ng/mL) or sf-αMEM alone, and were then incubated at 4°C overnight. Growth factor solutions were then removed and each well was washed with sf-αMEM prior to the addition of 1.5 mL of sf-αMEM to treatment wells, or 10% FCS, or osteogenic supplemented media (+OS media) to control wells.

5.2.3 Transwell™ Migration Assay

For full details of this method please refer to section 2.6. Sub-confluent cultures of SaOS-2 cells were passaged 1:1 the day prior to assay and serum-starved for 4 hr before harvesting and seeding of 2 x 10^5 cells into the upper chamber of 12 µm pore Transwells™ which had been pre-coated with various combinations of FN, IGFBP-5 and / or IGF-I. Following 5 hr incubation in a 5% CO_2/95 % air atmosphere at 37°C. Un-migrated cells were removed from the upper surface of the Transwell™ membrane with a cotton bud. Migrated cells were fixed in formaldehyde and stained with crystal violet. The Transwell™ membranes were immersed in a beaker of running tap water to remove excess stain and were allowed to air dry prior to extraction of adsorbed crystal violet dye in 10% acetic acid. Sub-samples were transferred to a 96-well microtitre plate and the absorbance read at 595 nm. Results are from 2 (-FN treatments) or 3 (+FN treatments) separate experiments with treatments assessed in triplicate. The data are expressed as the mean absorbance, as a percentage above the +VN control, ± the standard error of the mean (SEM).

5.2.4 Proliferation Assay

For full details of this method please refer to section 2.8. Sub-confluent cultures of SaOS-2 cells were serum-starved for 4 hr prior to harvest and seeding of 5000 cells / well into black 96-well plates containing various pre-bound combinations of VN, IGFBP-5 and IGF-I. Cells were allowed to proliferate for 24 hr, 48 hr or 72 hr prior to removal of spent media and storage of the culture plates at -80°C until analysis. Cyquant™ reagent was added to each well and the fluorescence measured at 480 nm excitation and 520 nm emission. Fluorescence units were converted to cell number

by using cells kept from the time of seeding to construct a cell number standard curve as per the manufacturer's instructions. Results are from 3 separate experiments performed in at least triplicate and are expressed as the mean cell number ± standard error of the means (SEM).

5.2.5 Solution Phase Culture

For full details of this method please refer to section 2.15. Sub-confluent cultures of SaOS-2 cells were washed twice with HBSS prior to harvest by trypsin / EDTA digestion and the addition of 1.44 x 10^5 cells/well in sf-αMEM into pre-prepared 6 well tissue culture plates (as per section 2.15) and incubation in a humidified 5% CO_2/95% air atmosphere at 37°C. Media was changed twice weekly using the protein concentrations detailed in section 2.15. Digital images were captured at various time-points through-out the culture period, as indicated in the figures and as detailed in section 2.12.

5.2.6 Differentiation Assay

For full details of this method please refer to section 2.16. Sub-confluent cultures of SaOS-2 cells were washed twice with HBSS prior to harvest by trysin / EDTA digestion and re-suspension in sf-αMEM. Cells were counted and seeded at 5 x 10^5 cells per well in a total volume of 3 mL. Plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 hr, or 7 days with 1 media change (for the 7 day plate) on day 4 using growth factor concentrations identical to those used at the time of seeding, described above. Digital photographs were taken to assess culture morphology prior to RNA extraction.

5.2.7 Real Time PCR Analysis

For full details of this method please refer to section 2.17. Monolayer cultures from differentiation assays at either 48 hr, or 7 days, were harvested with 1 mL Tri-Reagent (Sigma-Aldrich) and stored at -80°C until required for RNA extraction (less than 1 month). Total RNA was then isolated by addition of 0.2 mL of chloroform, then mixed and centrifuged at 12,000 x g for 15 minutes at 4°C, prior to the transfer of the aqueous phase to fresh 2 mL Eppendorf tubes and addition of 0.5 mL of 100% isopropanol with gentle mixing. Samples were then subjected to further

centrifugation at 12,000 x g for 10 min at 4°C prior to removal of the supernatant and washing of the RNA pellets with 1 mL 75% ethanol. Samples were gently mixed and centrifuged at 7500 x g for 5 min at 4°C prior to removal of the supernatant and brief air drying of the RNA pellets. Each pellet was resuspended in 20 µL of DEPC-treated ddH_20, transferred to fresh 0.5 mL Eppendorf tubes and immediately placed on ice. Total RNA concentration and quality of each sample was determined by UV spectrophotometry and were subsequently stored at -80°C until required for cDNA synthesis.

cDNA synthesis was performed on each total RNA sample using First Strand cDNA synthesis kit (AMV) according to the manufacturers instructions. Briefly, 0.5 µg of total RNA was reacted with 10 mM Tris, 50 mM KCl, 5 mM $MgCL_2$, 1 mM dNTPs, 3.2 µg of random hexamer oligonucleotide primers, 50 units 'Protector' RNase inhibitor (Roche), 20 units AMV reverse transcriptase and ddH_20 in a final volume of 20 µL. Reaction tubes were gently mixed, pulse centrifuged and incubated at 25°C for 10 min, 42°C for 60 min, 99°C for 5 min and finally 4°C o / n. The resulting cDNA samples were stored at -80°C until required for real time-PCR analysis.

Each cDNA sample was diluted 1:5 with TE buffer prior to the addition of 2.0 µL of diluted cDNA template to triplicate PCR reaction mixtures containing 10 µL of SYBR® green PCR master mix, 5 pmol of forward primer, 5 pmol of reverse primer and ddH_20 to a final volume of 20 µL per well. PCR microplates loaded with the PCR reaction mixtures were pulse-centrifuged, placed into an ABI Prism 7000 real time-PCR machine and subjected to 50°C for 2 min, 95.0°C for 10 min, then 40 cycles of 95.0°C for 15 sec and 60.0°C for 1 min, followed by a dissociation stage of 95.0°C for 15 sec, 60°C for 20 sec and 95°C for 15 sec. Raw data were exported to Microsoft Excel, transformed and analysed using the $2^{-\Delta\Delta C_T}$ method (User Bulletin # 2 for ABI Prism® 7000 Sequence Detection System and Livak and Schmittgen (Livak and Schmittgen 2001)). cDNA prepared as above from cells just prior to seeding (unseeded cells) was utilised as a time 0 control to which all treatments were compared. Results are expressed as mean fold change compared to the time zero (T_0) (un-seeded) cell control ± SEM from triplicate PCR reactions for each of 3

individual differentiation experiments. PCR primers for cbfa-1, ALP and COL-1 are presented in table 2.1.

5.3 RESULTS

5.3.1 Effect of pre-bound FN, IGFBP-5 and IGF-I on SaOS-2 cell migration.

In this series of experiments I wished to evaluate the migratory response of SaOS-2 cells exposed to the matrix protein FN, another ECM glycoprotein present in bone which has been shown to both interact with IGFBP-5 and modulate IGFBP-5 / IGF-I mediated cell responses (Xu *et al.* 2004). Here, I directly compared the response of SaOS-2 cells exposed to pre-bound combinations of FN ± IGFBP-5 ± IGF-I with results obtained for the VN-based treatments detailed in chapter 3 (Figure 5.1). FN was added in stoichiometrically equivalent amounts compared to that utilised for the previous VN studies, while the IGFBP-5 and IGF-I concentrations were kept equivalent. I observed that SaOS-2 cell migration was significantly enhanced in response to FN alone (147.7 ± 13.1% (n=9) of the +VN treatment) compared to the response to VN alone (100.0 ± 9.2% (n=9)) (p<0.05) (Figure 5.1). This migration was further enhanced in the additional presence of IGF-I (191.4 ± 14.4% (n=9) of the +VN treatment) compared to the migration response to VN alone (p<0.01), FN alone (p<0.05) and the corresponding VN / IGF-I treatment (126.8 ± 10.0% (n=9) of the +VN treatment) (p<0.01). This last result suggests that some IGF-I was retained on the Transwell™ membrane surface thus mediating increased SaOS-2 cell migration through the membrane (Fig 5.1). While, SaOS-2 cell migration in response to FN / IGFBP-5 (156.6 ± 15.3% (n=9) of the +VN treatment) was significantly higher than that measured for VN alone (p<0.01), and VN / IGFBP-5 (p<0.01) it was not different to that of FN alone. As was the case for the migration response to VN / IGFBP-5 / IGF-I, the FN / IGFBP-5 / IGF-I treatment (244.2 ± 17.3% (n=9) of the +VN treatment) produced a synergistic SaOS-2 cell migration response, and this was significantly higher than the response to VN alone (p<0.01), FN alone (p<0.01), FN / IGFBP-5 (p<0.01), FN / IGF-I (p<0.05) and VN / IGFBP-5 / IGF-I (p<0.05). As detailed in chapter 3 there was little or no migration of SaOS-2 cells in the absence of VN or FN (n=6) (Figure 5.1). Taken together, these data indicate that FN can induce enhanced SaOS-2 cell migration through 12 μm pore Transwell™ membranes, and that, as was the case for VN, the presence of IGFBP-5 and IGF-I

synergistically enhanced the migration response. The VN results from figure 3.1 have been included in light grey in figure 5.1 for comparative purposes.

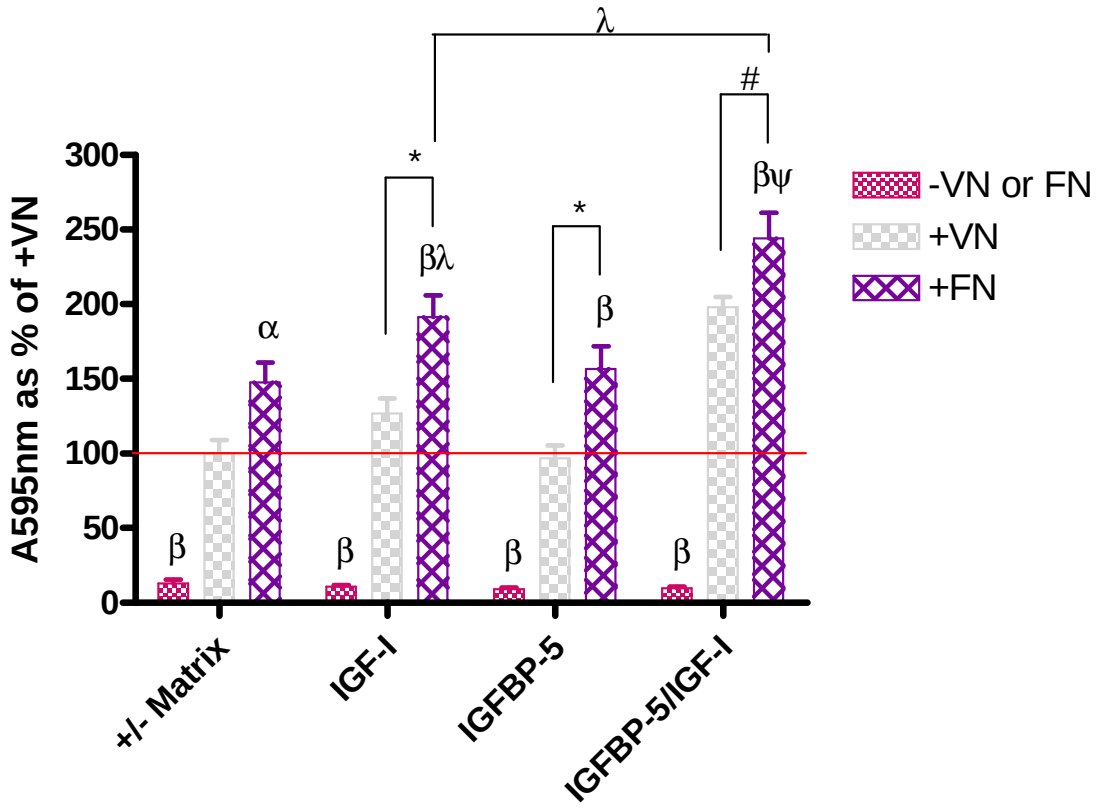


Figure 5.1. Effect of pre-bound FN, IGFBP-5 and IGF-I on SaOS-2 cell migration. SaOS-2 cells (2 x 10^5 cells/Transwell™) were incubated in serum free-αMEM and allowed to migrate for 5 hr on pre-bound combinations of IGFBP-5 (50 ng/well) and IGF-I (12.5 ng/well) in the presence or absence of pre-bound FN (6 µg/well). Following incubation, un-migrated cells were removed from the upper surface of the Transwell™ by cleaning with a cotton bud. Migrated cells were then fixed in formaldehyde for 20 min and subsequently stained with crystal violet for 20 min. Excess stain was removed by immersion of the inserts in a reservoir of circulating tap water and air dried prior to extraction of crystal violet in 0.5 mL of 10% acetic acid. Triplicate 100 µL sub-samples were then transferred to individual wells of a 96-well micro-titre plate and the absorbance read at 595 nm. Results were corrected using data from plate blanks (10% acetic acid alone) and are expressed as the corrected absorbance relative to the VN only control (100%) (horizontal red line) ± standard error of the means (SEM). Significant difference to the VN only control is indicated by α (p<0.05) or β (p<0.01) and was determined by post-hoc t-test. Significant difference between the FN only treatment and other FN groups is given by λ (p<0.05) or ψ (p<0.01) or select groups (where linked by line) is indicated by # (p<0.05) or * (p<0.01).

5.3.2 Effect of various doses of pre-bound FN on SaOS-2 cell proliferation.

In order to determine if FN alone could stimulate SaOS-2 cell proliferation in serum-free conditions a series of dose-response, time course assays were conducted (Figure 5.2). As was observed for the dose-dependent response of SaOS-2 cells to various concentrations of VN, detailed in chapter 3, there was no increase in cell number after 24 hr of serum-free culture in any treatment (n=6) above the number of cells seeded (5000 cells/well) (Figure 5.2a). After 48 hr, however, cell number in the 10% FCS control had increased to 6999 ± 212 cells (n=15), which was significantly higher than any other treatment (p<0.01) (Figure 5.2b). SaOS-2 cell number was generally maintained by tissue culture surfaces coated with FN, but was observed to be significantly decreased in the absence of FN (-FN control) (3606 ± 278 cells (n=15)), compared to SaOS-2 cells exposed to wells coated with FN at 48 μg/mL (5327 ± 432 cells (n=6)), 24 μg/mL (5049 ± 262 cells (n=15)), 12 μg/mL (5107 ± 314 cells (n=15)), 6 μg/mL (4685 ± 261 cells (n=15)), or 3 μg/mL (4701 ± 272 cells (n=15)) (p<0.01) and 1.5 μg/mL (4631 ± 287 cells (n=15)) (p<0.05) (Figure 5.2b). However, SaOS-2 cell number was not significantly different to the –FN control after 48 hr, when exposed to wells treated with either 0.75 μg/mL (n=9) or 0.375 μg/mL (n=9) FN. In addition, cell number in the 0.375 μg/mL treatment was significantly lower than cell numbers measured in wells treated with FN at 12 μg/mL, or above (p<0.05) (Figure 5.2b). This trend remained essentially unchanged after 72 hr with only the 10% FCS control (8442 ± 231 cells (n=12)) exhibiting an increased cell number, while numbers in the –FN control decreased further (2767 ± 193 cells (n=12)) (Figure5.2c). SaOS-2 cell population in cultures exposed to wells treated with 48 μg/mL (5454 ± 253 cells (n=6)), 24 μg/mL (5089 ± 199 cells (n=12)), 12 μg/mL (5193 ± 162 cells (n=12)), 6 μg/mL (4735 ± 109 cells (n=12)), or 3 μg/mL (4570 ± 178 cells (n=12)), 1.5 μg/mL (4438 ± 153 cells (n=12)) and 0.75 μgmL (4037 ± 240 cells (n=6)) remained significantly higher than the –FN control (p<0.05). Cell number was observed to decrease further in cultures exposed to wells coated with 0.375 μg/mL of FN (2945 ± 186 cells (n=6)), to levels similar to the –FN control. There was no significant difference between the results obtained for the treatments containing greater than 12 μg/mL of FN, although, cell numbers were significantly lower compared to the 12 μg/mL treatment in the 0.375 μg/mL, 0.75 μg/mL and 1.5 μg/mL treatments (p<0.01) and the 3 μg/mL and 6 μg/mL treatments (p<0.05) (Figure 5.2c). Taken together, these data demonstrate that pre-bound FN

does not stimulate SaOS-2 cell proliferation, but rather, may dose-dependently rescue SaOS-2 cell survival in serum-free conditions.

Figure 5.2. Effect of various doses of pre-bound FN on SaOS-2 cell proliferation. SaOS-2 cells (5000 cells/well) were incubated in serum free-αMEM and allowed to proliferate for a) 24 hr, b) 48 hr and c) 72 hr in wells pre-coated with 50 µL solutions of FN at various concentrations as indicated. Cells seeded into wells without VN, or in 10% FCS, were employed as controls. Following incubation, media was aspirated and the tissue culture plates were stored at -80°C. CyQUANT® reagent was then added to each well as per the manufacturer's instructions and the fluorescence measured at 480 nm excitation 520 nm emission. Results were corrected using data from plate blanks (CyQUANT® reagent only) and cell number calculated from a standard curve and expressed as mean cell number ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significance between treatments and the –FN control is indicated by α ($p<0.05$), or β ($p<0.01$). Significant difference to the 12 µg/mL treatment or select groups (where linked by line) is indicated by λ ($p<0.05$), or ψ ($p<0.01$). Significant difference to all other groups is indicated by * ($p<0.01$). Horizontal red line indicates initial number of cells seeded per well.

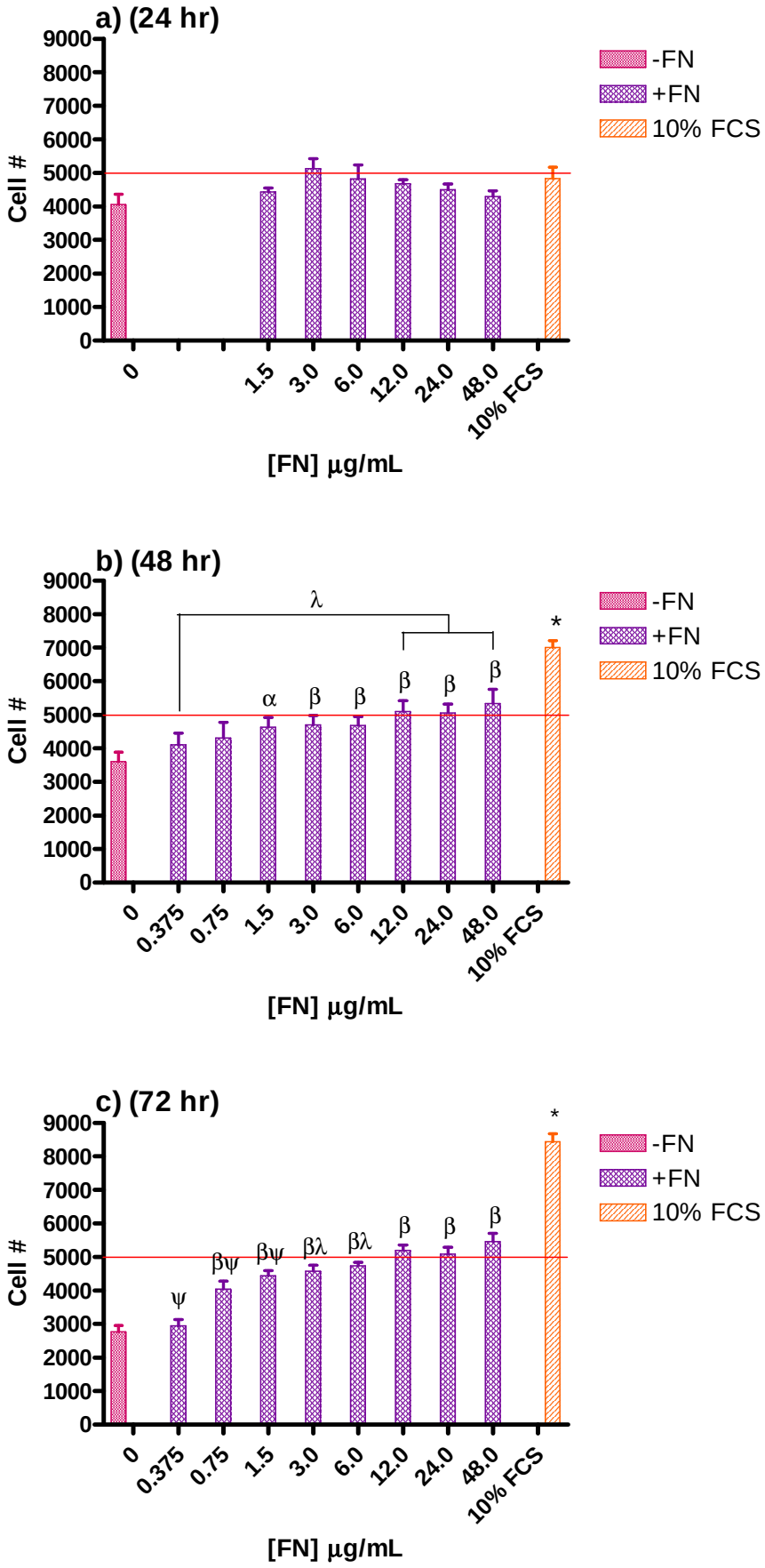

a) (24 hr)
Cell #
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
0
1.5
3.0
6.0
12.0
24.0
48.0
10% FCS
[FN] µg/mL
-FN
+FN
10% FCS
b) (48 hr)
Cell #
λ
*
α
β
β
β
β
β
0
0.375
0.75
1.5
3.0
6.0
12.0
24.0
48.0
10% FCS
[FN] µg/mL
-FN
+FN
10% FCS
c) (72 hr)
Cell #
*
ψ
βψ
βψ
βλ
βλ
β
β
β
0
0.375
0.75
1.5
3.0
6.0
12.0
24.0
48.0
10% FCS
[FN] µg/mL
-FN
+FN
10% FCS

Figure 5.2

5.3.3 Effect of various doses of pre-bound FN and VN on SaOS-2 cell proliferation.

Given that FN, like VN, is a significant constituent of serum (Yamada and Olden 1978) and is also present in bone matrix (Grzesik and Robey 1994), a series of FN dose response assays in the presence of 1 μg/mL of VN were performed to determine if the combined presence of both FN and VN could modulate a proliferative response in SaOS-2 cells, compared to either component in isolation. Similar to the results obtained for the individual components alone (Figures 3.3 and 5.2), after 24 hr no significant increase in cell number, over the initial cell seeding of 5000 cells/well was observed (n=6 for all treatments at 24 hr)(Figure 5.3a). There was no significant difference in cell number between the VN only control (5396 ± 392 cells) and the FN treatments at 1.5 μg/mL (4891 ± 259 cells), 3 μg/mL (4508 ± 175 cells), 6 μg/mL (4476 ± 296 cells) and the 10% FCS control (4831 ± 337 cells). There were, however, significantly fewer cells than the VN only control in wells where cells were cultured in wells coated with VN and 12 μg/mL (4181 ± 222 cells), 24 μg/mL (4407 ± 204 cells) or 48 μg/mL (4300 ± 205 cells) of FN (p<0.05)(Figure 5.3a). After 48 hr no significant difference in cell number was observed between any of the FN containing treatments and the VN only control (4831 ± 288 cells (n=15)) (Figure 5.3b). There were significantly fewer cells in wells coated with FN at 0.75 μg/mL (4158 ± 333 cells (n=9)) compared to wells coated with either 24 μg/mL (5155 ± 288 cells (n=15)) or 48 μg/mL (5183 ± 310 cells (n=6)) of FN (p<0.05). However, cell numbers in wells coated with FN at 0.375 μg/mL (4721 ± 230 cells (n=9)), 1.5 μg/mL (5052 ± 346 cells (n=15)), 3 μg/mL (4820 ± 324 cells (n=15)), 6 μg/mL (4575 ± 299 cells (n=15)) or 12 μg/mL (5006 ± 367 cells (n=15)) were not different to the 24, or 48 μg/mL treatments (Figure 5.3b). The cell population of the 10% FCS control (6999 ± 212 cells (n=15)) increased (as detailed in the previous section (section 5.3.2)) to a level significantly higher than all other treatments (p<0.01) (Figure 5.3b). Interestingly, after 72 hr cell numbers had increased in both the 10% FCS control (8442 ± 231 cells (n=12)) (as detailed in section 5.3.2) and in wells coated with FN at 48 μg/mL (6449 ± 179 cells (n=6)). The result obtained for the 48 μg/mL of FN treatment was significantly higher than both the VN only control (4802 ± 389 cells (n=12)) and all other FN containing wells (p<0.01), while the 10% FCS response was higher than all treatments (p<0.01) (Figure 5.3c). There was no difference in cell number between any of the FN containing treatments and the VN

only control ($n_{FN\ 0.375\ \mu g/mL,\ 0.75\ \mu g/mL} = 9$; $n_{FN1.5\ \mu g/mL,\ 3.0\ \mu g/mL,\ 6.0\ \mu g/mL,\ 12.0\ \mu g/mL,\ 24.0\ \mu g/mL} = 12$). Taken together these data indicate that the addition of FN and VN together do not substantially modify the proliferative response of SaOS-2 cells over a 72 hr period compared to either of the components alone, with perhaps the exception of the high dose (48 μg/mL) of FN.

Figure 5.3. Effect of various doses of pre-bound FN and VN on SaOS-2 cell proliferation. SaOS-2 cells (5000 cells/well) were incubated in serum free-αMEM and allowed to proliferate for a) 24 hr, b) 48 hr and c) 72 hr in wells pre-coated with 50 µL solutions of 1 µg/mL VN with FN at various concentrations as indicated. Cells seeded into wells coated with VN alone or in 10% FCS were employed as controls. Following incubation, media was aspirated and the tissue culture plates were stored at -80°C. CyQUANT® reagent was then added to each well as per the manufacturer's instructions and the fluorescence measured at 480 nm excitation and 520 nm emission. Results were corrected using data from plate blanks (CyQUANT® reagent only) and cell number calculated from a standard curve and expressed as mean cell number ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significant difference to the +VN control or select groups (where linked by line) is indicated by α ($p<0.05$) or β ($p<0.01$). Significant difference to all other groups is indicated by * ($p<0.01$). Horizontal red line indicates initial number of cells seeded per well.

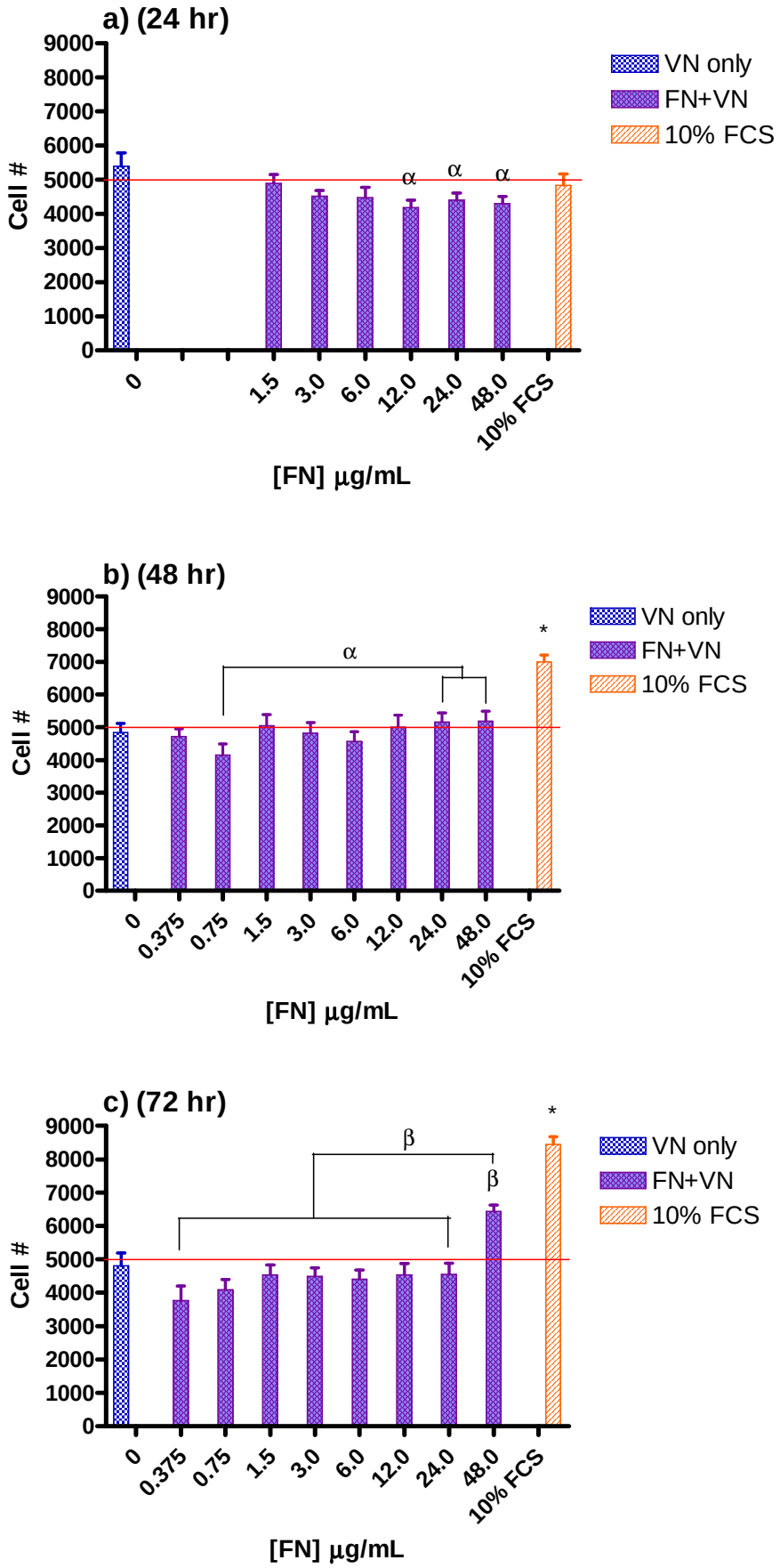

a) (24 hr)
Cell #
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
α
α
α
VN only
FN+VN
10% FCS
0
1.5
3.0
6.0
12.0
24.0
48.0
10% FCS
[FN] µg/mL
b) (48 hr)
Cell #
α
*
VN only
FN+VN
10% FCS
0
0.375
0.75
1.5
3.0
6.0
12.0
24.0
48.0
10% FCS
[FN] µg/mL
c) (72 hr)
Cell #
β
β
*
VN only
FN+VN
10% FCS
0
0.375
0.75
1.5
3.0
6.0
12.0
24.0
48.0
10% FCS
[FN] µg/mL

Figure 5.3

5.3.4 Effect of pre-bound FN, IGFBP-5 and IGF-I on SaOS-2 cell proliferation.

IGFBP-5 is known to bind to FN and this interaction has also been shown to modulate IGF-I mediated cell activity (Xu *et al.* 2004). Following the results of SaOS-2 cell migration studies, detailed in section 5.3.1, which demonstrated that SaOS-2 cell migration is synergistically enhanced in the presence of pre-bound FN, IGFBP-5 and IGF-I, I hypothesized that similarly pre-bound combinations of FN, IGFBP-5 and IGF-I could stimulate enhanced cell proliferation over the individual components alone. To test this hypothesis a series of proliferation assays in tissue culture plates coated with various combinations of FN (6 µg/mL), IGFBP-5 (1.6 µg/mL) and IGF-I (0.4 µg/mL) were performed. I observed that after 24 hr there had not been any increase in cell number in the VN only control (4927 ± 154 cells (n=18)) over the initial cell seeding density of 5000 cells/well. Furthermore, there was no significant difference in cell number between the VN only control and the –FN control (4427 ± 300 cells (n=17)), IGF-I only (4340 ± 326 cells (n=17)) or the IGFBP-5 / IGF-I (5037 ± 241 cells (n=17)) treatments. However, there were significantly fewer cells in the IGFBP-5 only treatment (3789 ± 246 cells (n=17)) compared to the VN only control ($p<0.01$) (Figure 5.4a). Conversely, cell numbers had increased above the initial cell seeding density in the 10% FCS control (6064 ± 143 cells), FN only (6011 ± 355 cells (n=18)), FN / IGF-I (6342 ± 359 cells (n=18)) and the FN / IGFBP-5 (6427 ± 401 cells (n=18)) treatments and were found to be significantly higher than the VN only control ($p<0.01$), as was the FN / IGFBP-5 / IGF-I (6170 ± 469 cells (n=18)) treatment, although only slightly less so ($p<0.05$) (Figure 5.4a). After 48 hr, cell numbers in the –FN (3327 ± 159 cells (n=18)), IGF-I only (4354 ± 232 cells (n=18)), IGFBP-5 only (3414 ± 127 cells (n=18)) and the IGFBP-5 / IGF-I treatments had significantly fewer cells than the VN only control (5589 ± 174 cells (n=18)) ($p<0.01$). This was mainly due to a slight increase in cell number in the VN only control in contrast to the general decrease in cell number in the treatments without FN. Cell numbers on the other hand increased in wells which had been pre-coated with FN alone (6395 ± 209 cells (n=18)), FN / IGF-I (7737 ± 416 cells (n=18)), FN / IGFBP-5 (7006 ± 261 cells (n=18)), FN / IGFBP-5 / IGF-I (7193 ± 419 cells (n=18)) or exposed to 10% FCS (8163 ± 148 cells (n=18)). All of these treatments led to cell numbers which were significantly greater than the VN only control ($p<0.01$). Furthermore, the 10% FCS control samples contained significantly more cells than wells coated with FN only ($p<0.01$), FN / IGFBP-5

(p<0.01), or FN / IGFBP-5 / IGF-I (p<0.05), but not FN / IGF-I (Figure 5.4b). After 72 hr, cell numbers in the VN only control (5382 ± 297 cells (n=18)) had not changed from that measured after 48 hr, while cell numbers in the –FN control (2574 ± 135 cells (n=18)), IGF-I only (2969 ± 120 cells (n=18)), IGFBP-5 (2601 ± 94 cells (n=18)) and IGFBP-5 / IGF-I (3232 ± 124 cells (n=18)) had decreased further compared to the VN only control (p<0.01). In addition, cells exposed to wells coated with FN only (4720 ± 224 cells (n=18)), FN / IGF-I (5354 ± 243 cells (n=18)), FN / IGFBP-5 (5386 ± 211 cells (n=18)) and FN / IGFBP-5 / IGF-I (6133 ± 274 cells (n=18)) decreased in number to levels equivalent to that of the VN only control. Intriguingly, SaOS-2 populations in wells coated with FN / IGFBP-5 remained significantly greater in cell numbers in the FN only control (p<0.05), while, cell numbers in wells coated with FN / IGFBP-5 / IGF-I remained higher than the cell number measured in wells coated with FN alone (p<0.01), or FN / IGF-I or FN / IGFBP-5 (p<0.05). The only treatment that lead to an increase in cell number between 48 hr and 72 hr, however, was the 10% FCS control (8917 ± 143 cells (n=18)) which had a higher cell number than any other treatment (p<0.01) (Figure 5.4c). Taken together, these data support the findings reported in section 5.3.2 that FN alone can support cell survival over 72 hr, but does not support a sustained proliferative response over the same time frame. Furthermore, these data suggest that pre-binding of IGFBP-5 and IGF-I together with FN can reduce the rate of decline in cell number compared to FN alone, or in combination with either IGFBP-5, or IGF-I, over 72 hr.

Figure 5.4. Effect of pre-bound FN, IGFBP-5 and IGF-I on SaOS-2 cell proliferation. SaOS-2 cells (5000 cells/well) were incubated in serum-free-αMEM and allowed to proliferate for a) 24 hr, b) 48 hr and c) 72 hr in wells pre-coated with solutions of IGFBP-5 (69.5 ng/well) and/or IGF-I (17.4 ng/well) in the presence or absence of pre-bound FN (6 µg/mL). Cells seeded into wells without FN or in 10% FCS were employed as controls. Following incubation, media was aspirated and the tissue culture plates were stored at -80°C. CyQUANT® reagent was then added to each well, as per manufacturer's instructions, and the fluorescence measured at 480 nm excitation 520 nm emission. Results were corrected using data from plate blanks (CyQUANT® reagent only), cell number calculated from a cell number standard curve and expressed as mean cell number ± standard error of the means (SEM). Statistical significance was determined by post-hoc t-test. Significant difference to the +VN control or select groups (where linked by line) is indicated by α ($p<0.05$) or β ($p<0.01$). Significant difference to the 10% FCS control is indicated by # ($p<0.05$) or * ($p<0.01$). Significant difference to the +FN control is indicated by λ ($p<0.05$) or ψ ($p<0.01$). Horizontal red line indicates initial number of cells seeded per well.

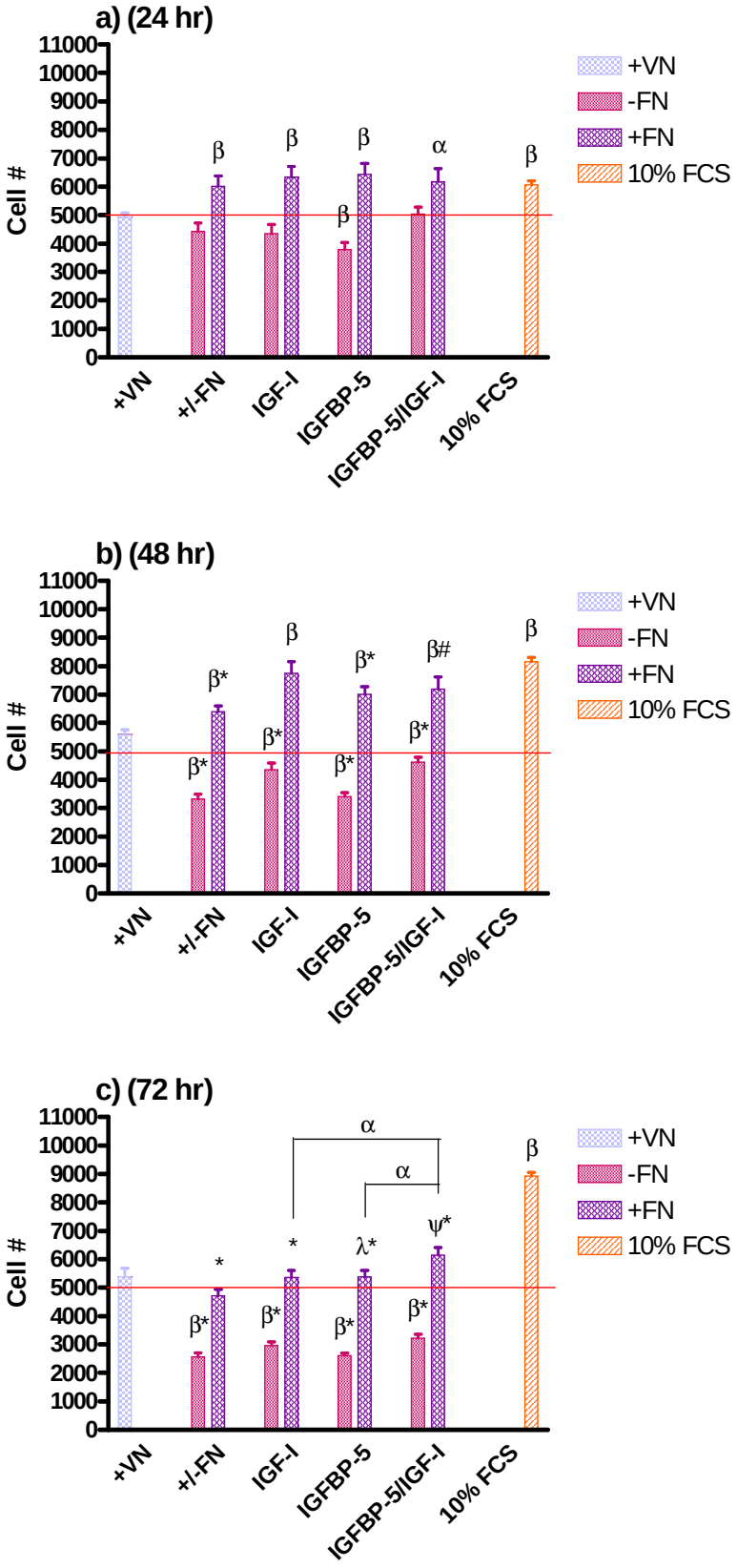

a) (24 hr)
b) (48 hr)
c) (72 hr)
Cell #
11000
10000
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
+VN
-FN
+FN
10% FCS
+/-FN
IGF-I
IGFBP-5
IGFBP-5/IGF-I
10% FCS

Figure 5.4

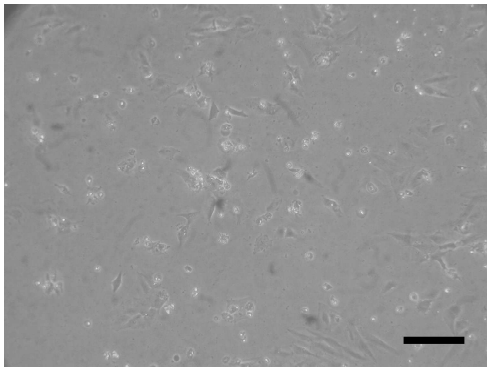
5.3.5 Effect of solution phase VN and FN with or without EGF or bFGF on SaOS-2 cell culture morphology.

In order to determine if solution phase VN and FN could sustain SaOS-2 cell cultures over longer periods of time than 72 hr, I propagated SaOS-2 cells in the presence of VN and FN with, or without, the addition of EGF, or bFGF, or both, and examined culture morphology. After 12 days of continuous culture with re-feeding twice weekly, SaOS-2 cells maintained in the presence of VN generally appeared irregular in shape with small cytoplasms, were poorly spread and some cells exhibited blebbing. In addition, there were an obvious number of dead and floating rounded cells. Those cells which did appear to be well attached to the culture surface were clustered tightly into small groups (Figure 5.5a). In contrast, SaOS-2 cells cultured in the presence of FN, while also small and irregularly shaped with a number of dead and apoptotic cells present, were more numerous and more evenly distributed over the culture surface than those exposed to VN alone (Figure 5.5b). Interestingly, the addition of the VN and FN together resulted in a more elongated cell morphology and more attached cells than either of the components in isolation (Figure 5.5c). Similarly, cells cultured in the presence of VN, FN and EGF had greatly improved cell and culture morphology with many more attached and well spread cells of a generally elongated morphology. In addition, there were fewer dead, or floating, cells present compared to cultures exposed to VN, or FN alone, or in combination. Cells cultured in the presence of VN and FN continued to exhibit a number of cells with apoptotic morphology (Figure 5.5d). SaOS-2 cells cultured in the presence of VN, FN and bFGF, were in general very small, cuboidal-shaped cells with very little cytoplasm. There also appeared to be fewer attached cells compared to the VN, FN, EGF treatment (Figure 5.5e). When SaOS-2 cells were exposed to all four components, ie. VN, FN, EGF and bFGF, cell morphology assumed a more heterogeneous phenotype. Some cells adopted the more elongated shape evident in the VN, FN, EGF treatment, while others were smaller and more cuboidal in shape, similar to those in the VN, FN, bFGF treatment (Figure 5.5f). In comparison, SaOS-2 cells cultured in 10% FCS in a parallel study were confluent, small and had a generally cuboidal morphology, typical of SaOS-2 cells cultured for 10 – 14 days (Fig 5.5g – h). These data indicated that neither VN, nor FN, in isolation, could sustain SaOS-2 cell viability. However, when used in combination, and with the addition of EGF, SaOS-2 cell cultures generally appeared healthier with more

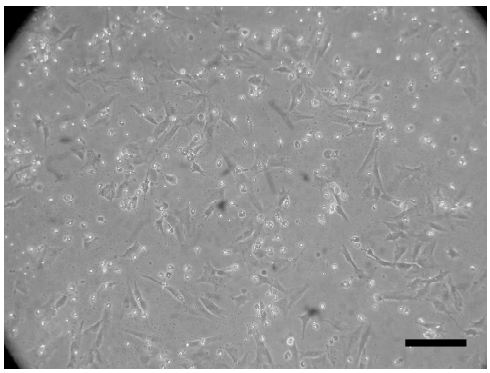
homogeneous cell morphologies than the other treatments after 12 days of culture, but did not attain the extent of growth observed when SaOS-2 cells were cultured in the presence of 10% FCS.

Figure 5.5. Effect of solution phase VN and FN with or without EGF or bFGF on SaOS-2 cell culture morphology.
Sub-confluent cultures of SaOS-2 cells were harvested using trypsin / EDTA digestion prior to the addition of 1.44 x 10^5 cells/well into pre-prepared 6-well tissue culture plates containing solutions of VN / sf-αMEM (1 µg/mL), FN / sf-αMEM (6 µg/mL) or VN / FN / sf-αMEM (1 µg/mL / 6 µg/mL). Cells were added to individual wells of a 6 well tissue culture plate together with either EGF (5 ng/mL), or bFGF (5 ng/mL), as indicated. The plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37 °C for up to 12 days. Spent culture media was changed twice weekly using protein concentrations detailed above and in section 2.15. Digital images of each culture were taken at X100 magnification on day 12 (or days 10 and 14 for 10% FCS cultures) as indicated. Bar = 200µm.
(Note: 10% FCS images were reproduced with permission of K, Shrobback, Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology).

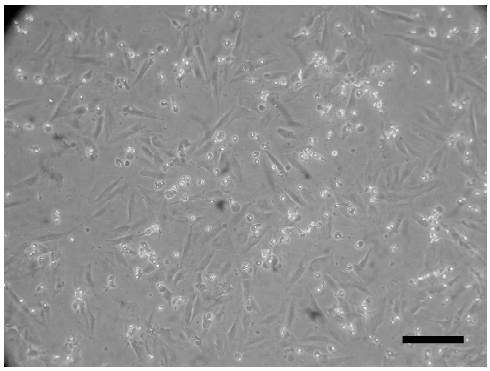
x100



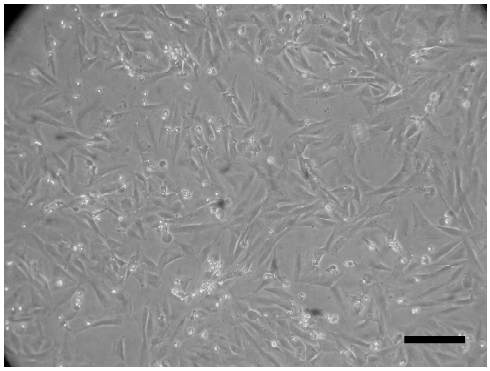
a) VN



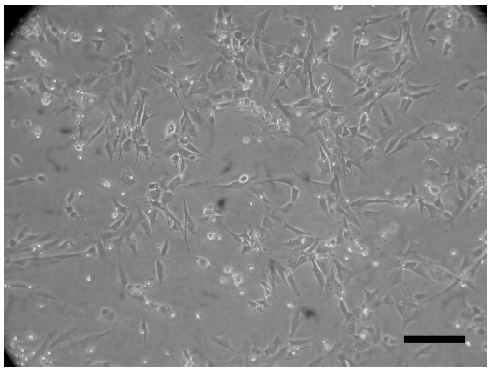
b) FN



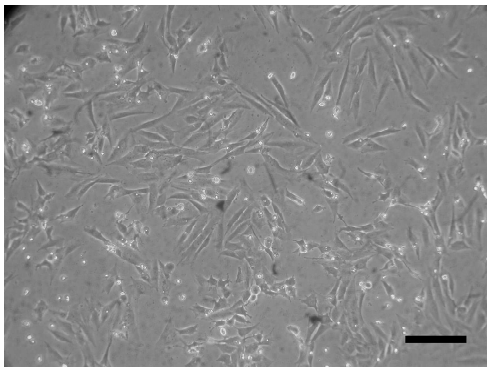
c) VN/FN



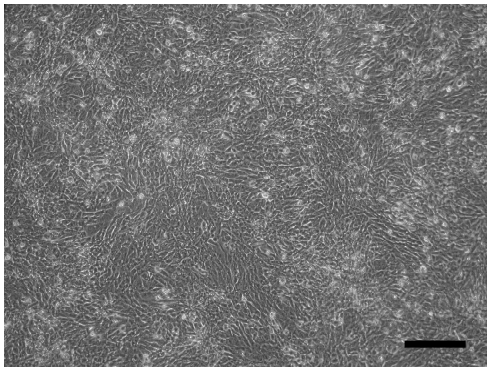
d) VN/FN/EGF



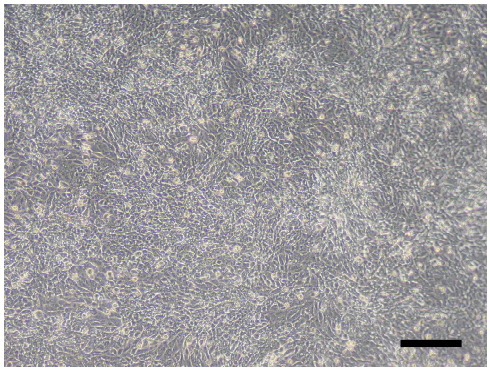
e) VN/FN/bFGF



f) VN/FN/EGF/bFGF



g) 10% FCS (day 10)



h) 10% FCS (day 14)

Figure 5.5

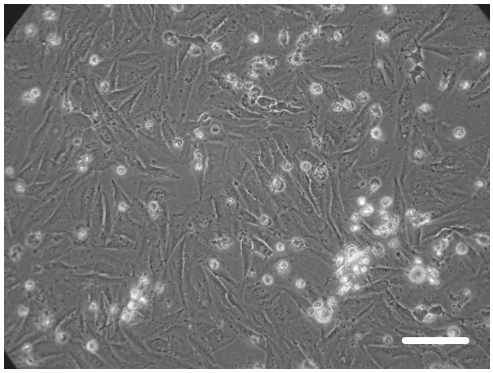
5.3.6 Effect of VN, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF on SaOS-2 cell morphology, compared to osteoblastic differentiation media.

Osteoblasts often change their morphology during osteoblastic cell differentiation (Aubin 1998). Therefore, I monitored the change in cell morphology of SaOS-2 cells exposed to VN alone, VN / IGFBP-5 / IGF-I, VN / TGF-β_1, or VN / FN / EGF, over 7 days compared to SaOS-2 cells exposed to 10% FCS, or 10% FCS supplemented with known osteogenic agents (+OS media), as part of a broader study into the effect of these treatments on osteoblast marker expression. SaOS-2 cells were seeded at high density into 6 well plates which had been pre-prepared with the matrix proteins and growth factors. After 48 hr SaOS-2 cell cultures exposed to 10% FCS, or +OS media were 100% confluent and these cells had adopted a small cobblestone like morphology, indicative of osteoblast-like differentiation, although some cells exhibited mitotic indices (Figure 5.6a.5 & 6). In contrast SaOS-2 cell cultures exposed to VN alone, VN / IGFBP-5 / IGF-I, or VN / TGF-β_1, were not confluent and cell morphology was both irregular and heterogeneous. In addition, a few cells exhibited apoptotic-like morphologies as assessed by visualisation of cell membrane blebbing, while a few others had visible mitotic indices (Figure 5.6a.1-3). Interestingly, the VN / FN / EGF treatment induced the elongated, fibroblast like morphology which was evident in the solution phase cultures detailed above in section 5.3.5 and Figure 5.5d. Furthermore, SaOS-2 cell cultures exposed to this treatment were confluent and had few cells with apoptotic type morphologies, but also very few visible mitotic indices (Figure 5.6a.4). After 7 days SaOS-2 cell cultures exposed to VN alone, and VN / TGF-β_1, had visibly decreased in cell number, although the morphologies of cells exposed to these treatments remained largely unchanged from that described above after 48 hr, albeit with fewer cells (Figure 5.6b.1 & 3). Similarly, SaOS-2 cell cultures exposed to the VN / FN / EGF treatment were still confluent, while the cells themselves remained relatively elongated (Figure 5.6b.4). Of particular interest, however, SaOS-2 cell cultures exposed to the VN / IGFBP-5 / IGF-I treatment appeared almost confluent and the cell morphology had changed from irregular shaped, as described above, to a more cobblestone type morphology similar to that of cells exposed to the 10% FCS treatment (Figure 5.6b.2 & 5). The +OS media induced similar cell morphology and density to the 10% FCS treatment but also induced the formation of bright refractory

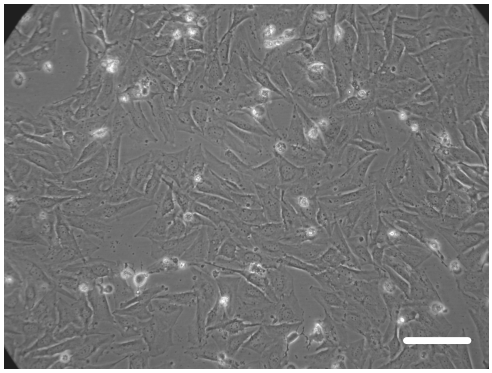
bodies (Figure 5.6b.6). I have previously identified these refractory bodies in this cell line to be calcium deposits by Von Kossa staining (data not shown).

Figure 5.6a Effect of VN, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF on SaOS-2 cell morphology after 48 hr. Sub-confluent cultures of SaOS-2 cells were harvested with trypsin / EDTA digestion prior to the addition of 5 x 10^5 cells/well in pre-prepared 6 well tissue culture plates coated with solutions of VN / sf-αMEM (1 µg/mL), VN / FN / sf-αMEM (1 µg/mL / 6 µg/mL) IGFBP-5 / IGF-I / sf-αMEM (160 ng/mL / 40 ng/mL), TGF-β_1 / sf-αMEM (115.2 ng/mL), or EGF / sf-αMEM (115.2 ng/mL) in combinations as indicated above and as detailed in section 5.2.2 and 2.16. Plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 hr. SaOS-2 cells cultured in the presence of 10% FCS, or osteogenic supplemented media (+OS media), were utilised as controls. Digital images were taken at X200 magnification to assess culture morphology prior to total RNA extraction. Bar = 100 µm.

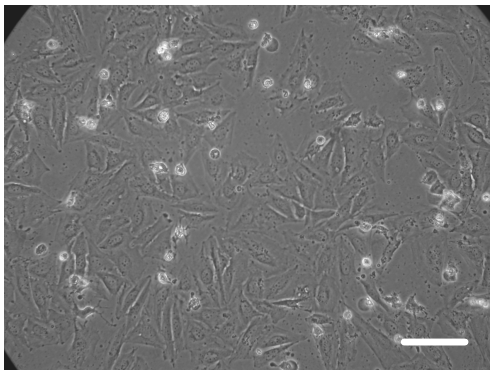
x200



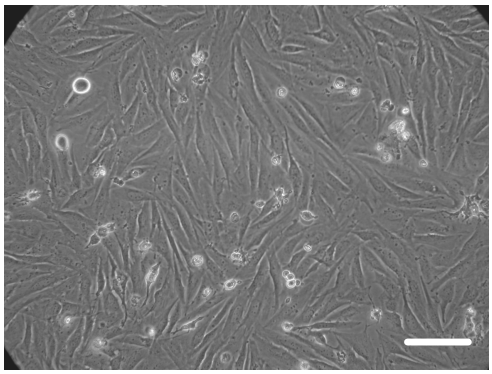
a.1. VN



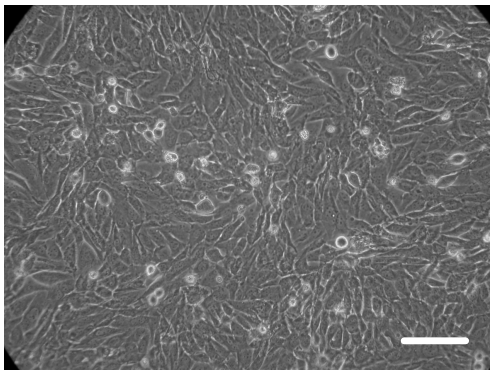
a.2. VN/IGFBP-5/IGF-I



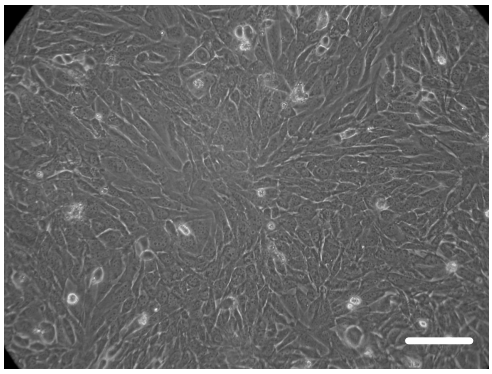
a.3. VN/TGF-ß$_1$



a.4. VN/FN/EGF



a.5. 10% FCS

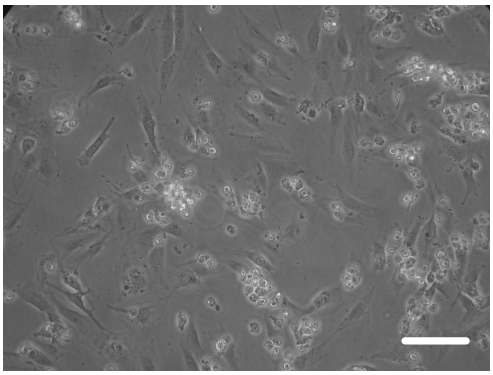


a.6. +OS

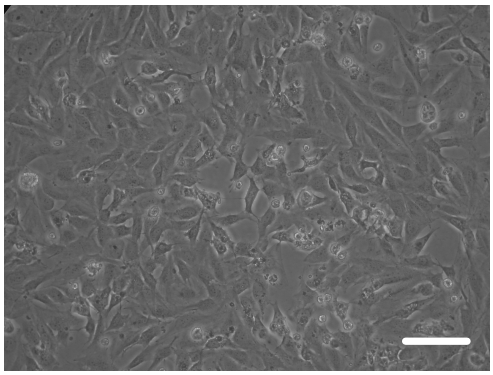
Figure 5.6a

Figure 5.6b Effect of VN, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF on SaOS-2 cell morphology after 7 days. Sub-confluent cultures of SaOS-2 cells were harvested with trypsin / EDTA digestion prior to the addition of 5 x 10^5 cells/well in pre-prepared 6 well tissue culture plates coated with solutions of VN / sf-αMEM (1 µg/mL), VN / FN / sf-αMEM (1 µg/mL / 6 µg/mL) IGFBP-5 / IGF-I / sf-αMEM (160 ng/mL / 40 ng/mL), TGF-β_1 / sf-αMEM (115.2 ng/mL), or EGF / sf-αMEM (115.2 ng/mL) in combinations as indicated above and as detailed in section 5.2.2 and 2.16. Plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 7 days with a single media change on day 4 using growth factor concentrations as described above. SaOS-2 cells cultured in the presence of 10% FCS, or osteogenic supplemented media (+OS media), were utilised as controls. Digital images were taken at X200 magnification to assess culture morphology prior to total RNA extraction. Bar = 100 µm.

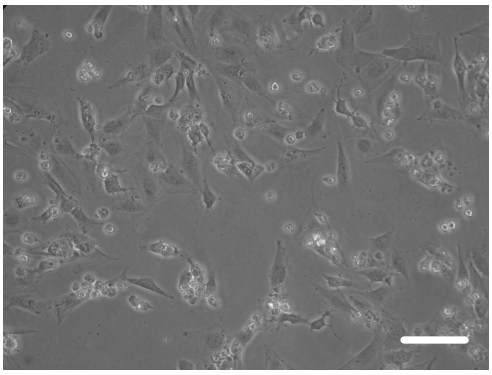
x200



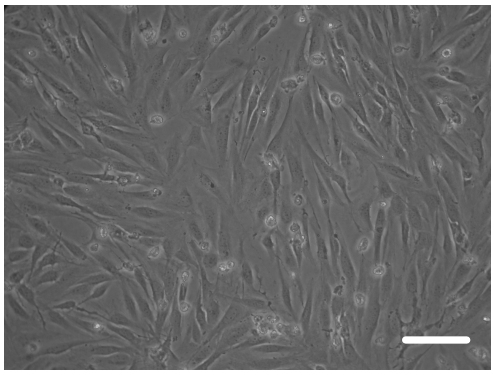
b.1. VN



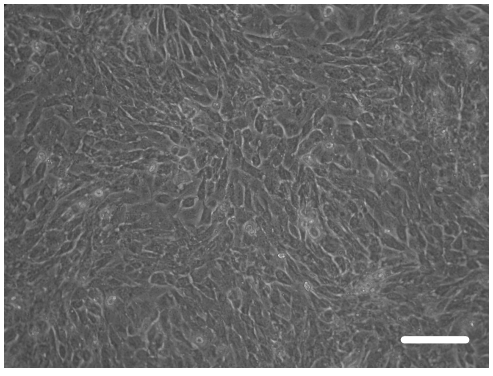
b.2. VN/IGFBP-5/IGF-I



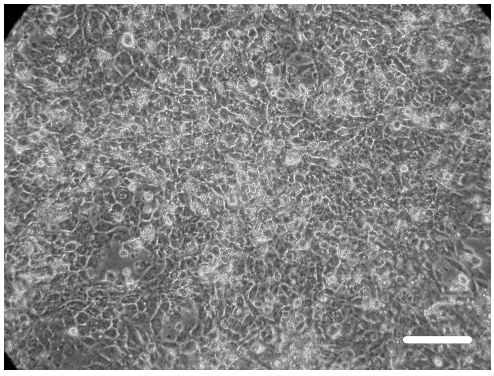
b.3. VN/TGF-ß$_1$



b.4. VN/FN/EGF



b.5. 10% FCS



b.6. +OS

Figure 5.6b

5.3.7 Effect of VN, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF on osteoblast differentiation marker expression in SaOS-2 cells.

VN has been shown to induce type I collagen expression in human mesenchymal stem cells (Salasznyk *et al.* 2004b). In addition, IGF-I has been implicated in bone formation processes in vivo and in vitro (Mohan and Baylink 1991; 1993; 1996; Long *et al.* 1998; Mohan and Baylink 1999; Jia and Heersche 2000; Yakar *et al.* 2002) while, IGFBP-5 over expression *in vitro* has been associated with the attenuation of various osteoblast differentiation markers (Durant *et al.* 2004). In view of this and the reasons outlined above in sections 5.3.5 and 5.3.6, I assessed osteoblast differentiation marker expression in SaOS-2 cells in the presence of VN alone, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF. I found that after 48 hr there had been no change in cbfa-1 mRNA transcript expression compared to the time zero (T_0) control in any of the treatments, with the exception of the 10% FCS control which induced a 4.11 ± 1.11 fold increase in cbfa-1 expression compared to the T_0 control (p0.05) (n=3 for all treatments) (Figure 5.7a). ALP transcript expression was increased by 3.99 ± 0.65 fold (VN / IGFBP-5 / IGF-I), 2.99 ± 0.42 fold (VN / TGF-β_1) or 3.00 ± 0.36 fold (VN / FN / EGF) compared to the T_0 control (p<0.01). Similarly both the 10% FCS control and the +OS media induced a 5.35 ± 1.27 fold and 2.50 ± 0.48 fold increase in SaOS-2 cell ALP mRNA expression respectively (p<0.05). However, ALP expression in response to VN only (2.4 ± 0.61 fold) was not significantly different to the T_0 control (Figure 5.7b). While Col-I expression was not significantly changed by any treatment compared to the T_0 control (1.00 ± 0.07 fold change), there was a significantly higher relative expression level of Col-I transcripts in the VN / FN / EGF treatment (1.41 ± 0.16 fold increase above the T_0 control) and the +OS treatment (0.82 ± 0.14 fold of the T_0 control) (p<0.05) (Figure 5.7c).

In a parallel series of experiments, the cells were cultured for 7 days with media changes for each treatment occurring on day 4, prior to RNA extraction, subsequent cDNA synthesis and real-time PCR. After 7 days of culture no significant difference in cbfa-1 mRNA transcript expression was observed between any of the individual treatments and the T_0 control. Nor were any differences observed between any of the treatments, including either the 10% FCS control or the +OS treatment (n=3 for all treatments) (Figure 5.8a). Conversely, after 7 days of culture ALP transcript

expression in the VN only (0.81 ± 0.23 fold of the T_0 control), VN / TGF-β_1 (1.00 ± 0.09 fold of the T_0 control) and the VN / FN / EGF (0.97 ± 0.01 fold of the T_0 control) were decreased to levels equivalent to the T_0 control. While, the ALP transcript levels in the VN / IGFBP-5 / IGF-I (2.35 ± 0.43 fold of the T_0 control), 10% FCS control (4.06 ± 1.29 fold of the T_0 control) and the +OS treatment (2.15 ± 0.37 fold of the T_0 control) were relatively lower at 7 days compared to the same treatments at 48 hr, ALP transcript levels in the VN / IGFBP-5 / IGF-I and the +OS treatment remained significantly higher than ALP levels in the T_0 control ($p<0.05$). While the mean relative level of ALP transcript in cells exposed to the 10% FCS control was higher than all other treatments, there was no statistical difference between this treatment and ALP transcript levels in the T_0 control. This was likely due to the large error associated with this treatment (Figure 5.8b). Surprisingly, Col-I transcript levels were lower in all treatments after 7 days compared to the same treatments after 48 hr, although Col-I transcript levels in cells exposed to VN only (0.70 ± 0.2 fold of the T_0 control), VN / FN / EGF (0.98 ± 0.13 fold of the T_0 control) and the 10% FCS control (0.54 ± 0.18 fold of the T_0 control) remained statistically equivalent to the T_0 control. In addition, the relative level of Col-I transcripts in cells exposed to VN / IGFBP-5 / IGF-I (0.52 ± 0.09 fold of the T_0 control), VN / TGF-β_1 (0.60 ± 0.06 fold of the T_0 control) and the +OS treatment (0.31 ± 0.04 fold of the T_0 control) was significantly lower than the level of Col-I transcripts in the T_0 control (1.00 ± 0.07 fold of the T_0 control) ($p<0.01$). Furthermore, relative Col-I transcript levels in cells exposed to VN / IGFBP-5 / IGF-I and VN / TGF-β_1 were significantly lower than in cells exposed to VN / FN / EGF ($p<0.05$), as were Col-I transcript levels in cells exposed to the +OS treatment ($p<0.01$) (Figure 5.8c). Taken together these data suggest that neither VN alone, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF had any effect on cbfa-1 mRNA expression after 48 hr or 7 days. However, the VN / IGFBP-5 / IGF-I treatment was able to increase ALP mRNA transcription in the absence of serum in the short term and maintained a higher level of ALP transcription after 7 days of culture compared to any of the other serum-free treatments. Somewhat surprisingly, the +OS treatment appeared to have down regulated Col-I expression after 48 hr, while the VN only, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 and VN / FN / EGF treatments did not significantly affect Col-I transcript levels at 48 hr. The relatively low levels of Col-I mRNA transcripts measured in cells exposed to VN only, VN / IGFBP-5 / IGF-I

Figure 5.7. Effect of VN, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF on osteoblast differentiation marker expression in SaOS-2 cells after 48 hr. Sub-confluent cultures of SaOS-2 cells were harvested using trypsin / EDTA digestion prior to the addition of 5 x 10^5 cells/well in pre-prepared 6 well tissue culture plates coated with solutions of VN / sf-αMEM (1 µg/mL), VN / FN / sf-αMEM (1 µg VN/mL / 6 µg FN/mL), IGFBP-5 / IGF-I / sf-αMEM (160 ng BP-5/mL / 40 ng IGF-I/mL), TGF-β_1 / sf-αMEM (115.2 ng/mL), or EGF / sf-αMEM (115.2 ng/mL) in combinations as indicated above and as detailed in section 5.2.2 and 2.16. SaOS-2 cells cultured in the presence of 10% FCS or osteogenic supplemented media (+OS media) were utilised as controls. Plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 48 hr prior to total RNA extraction and subsequent cDNA synthesis. Sample cDNA was subjected to real-time PCR using PCR primers for a) cbfa-1, b) ALP or c) COL-1 $\alpha_1$chain. Results were normalized against 18s mRNA expression and are presented as mean fold change from time 0 (T_0) control (cDNA derived from un-seeded cells and indicated by horizontal red line) ± standard error of the means (SEM) from triplicate PCR reactions for each of 3 individual differentiation experiments. Significant differences between individual treatments and the T_0 control, or between treatments linked by line, was determined by post-hoc t-test and is indicated by α (p<0.05) or β (p<0.01).

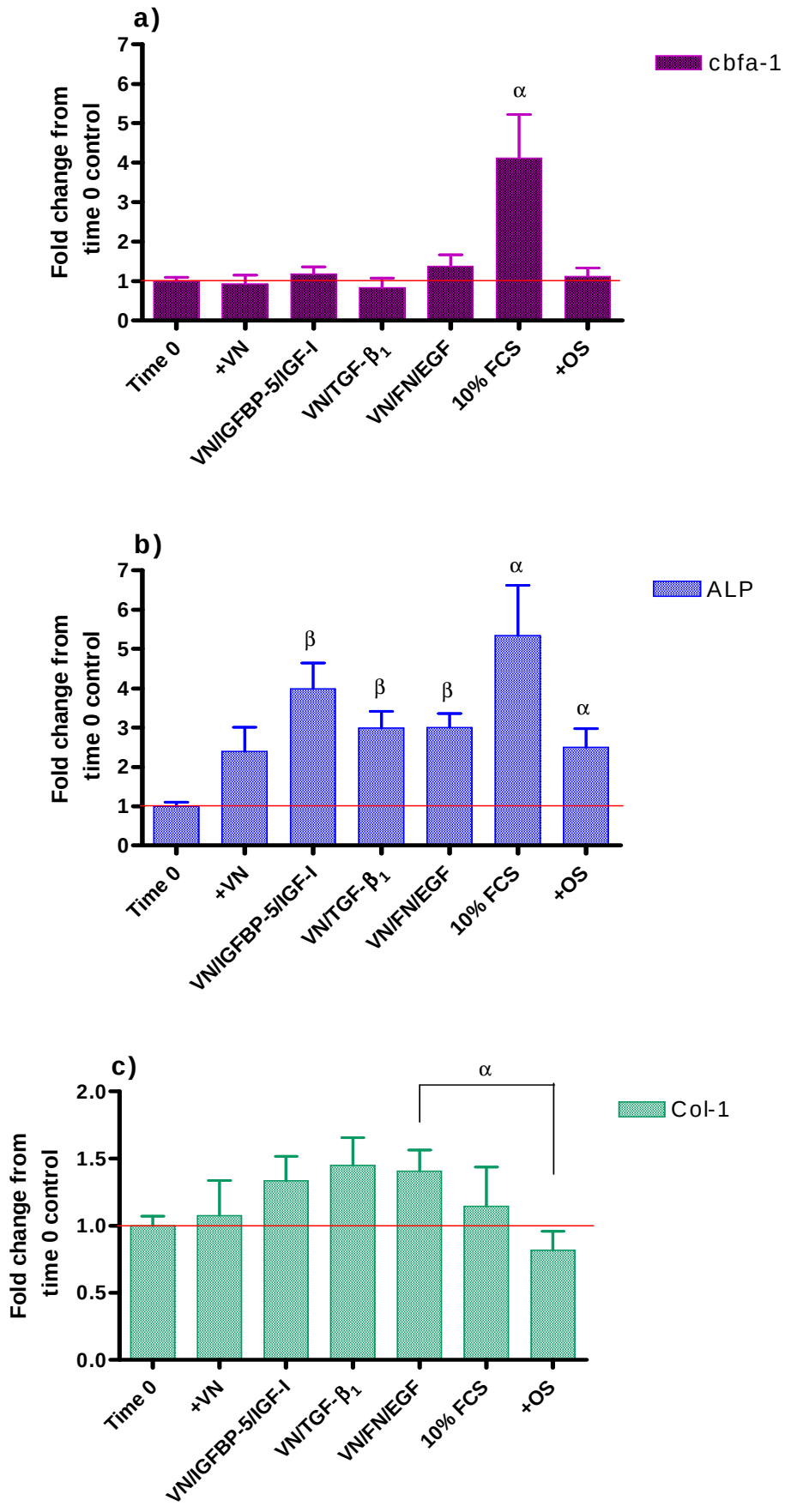

a)
Fold change from time 0 control
cbfa-1
α
Time 0
+VN
VN/IGFBP-5/IGF-I
VN/TGF-β1
VN/FN/EGF
10% FCS
+OS
b)
Fold change from time 0 control
ALP
α
β
β
β
α
Time 0
+VN
VN/IGFBP-5/IGF-I
VN/TGF-β1
VN/FN/EGF
10% FCS
+OS
c)
Fold change from time 0 control
Col-1
α
Time 0
+VN
VN/IGFBP-5/IGF-I
VN/TGF-β1
VN/FN/EGF
10% FCS
+OS

Figure 5.7

Figure 5.8. Effect of VN, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 or VN / FN / EGF on osteoblast differentiation marker expression in SaOS-2 cells after 7 days. Sub-confluent cultures of SaOS-2 cells were harvested using trypsin / EDTA digestion prior to the addition of 5 x 10^5 cells/well in pre-prepared 6 well tissue culture plates coated with solutions of VN / sf-αMEM (1 µg/mL), VN / FN / sf-αMEM (1 µg VN/mL / 6 µg FN/mL) IGFBP-5 / IGF-I / sf-αMEM (160 ng BP-5/mL / 40 ng IGF-I/mL), TGF-β_1 / sf-αMEM (115.2 ng/mL), or EGF / sf-αMEM (115.2 ng/mL) in combinations as indicated above and as detailed in section 5.2.2 and 2.16. SaOS-2 cells cultured in the presence of 10% FCS, or osteogenic supplemented media (+OS media) were utilised as controls. Plates were incubated in a humidified 5% CO_2/95% air atmosphere at 37°C for 7 days with a single media change at day 4, prior to total RNA extraction and subsequent cDNA synthesis. Sample cDNA was subjected to real time PCR using PCR primers for a) cbfa-1, b) ALP or c) COL-1 α_1 chain. Results were normalized against 18s mRNA expression and are presented as mean fold change from time zero (T_0) control (cDNA derived from unseeded cells and indicated by horizontal red line) ± SEM from triplicate PCR reactions for each of 3 individual differentiation experiments. Significant differences between individual treatments and the T_0 control, or between treatments linked by line, were determined by post-hoc t-test and is indicated by α (p<0.05) or β (p<0.01). Significant difference between the VN / IGFBP-5 / IGF-I treatment and all other serum-free VN containing treatments is given by ψ (p<0.05).

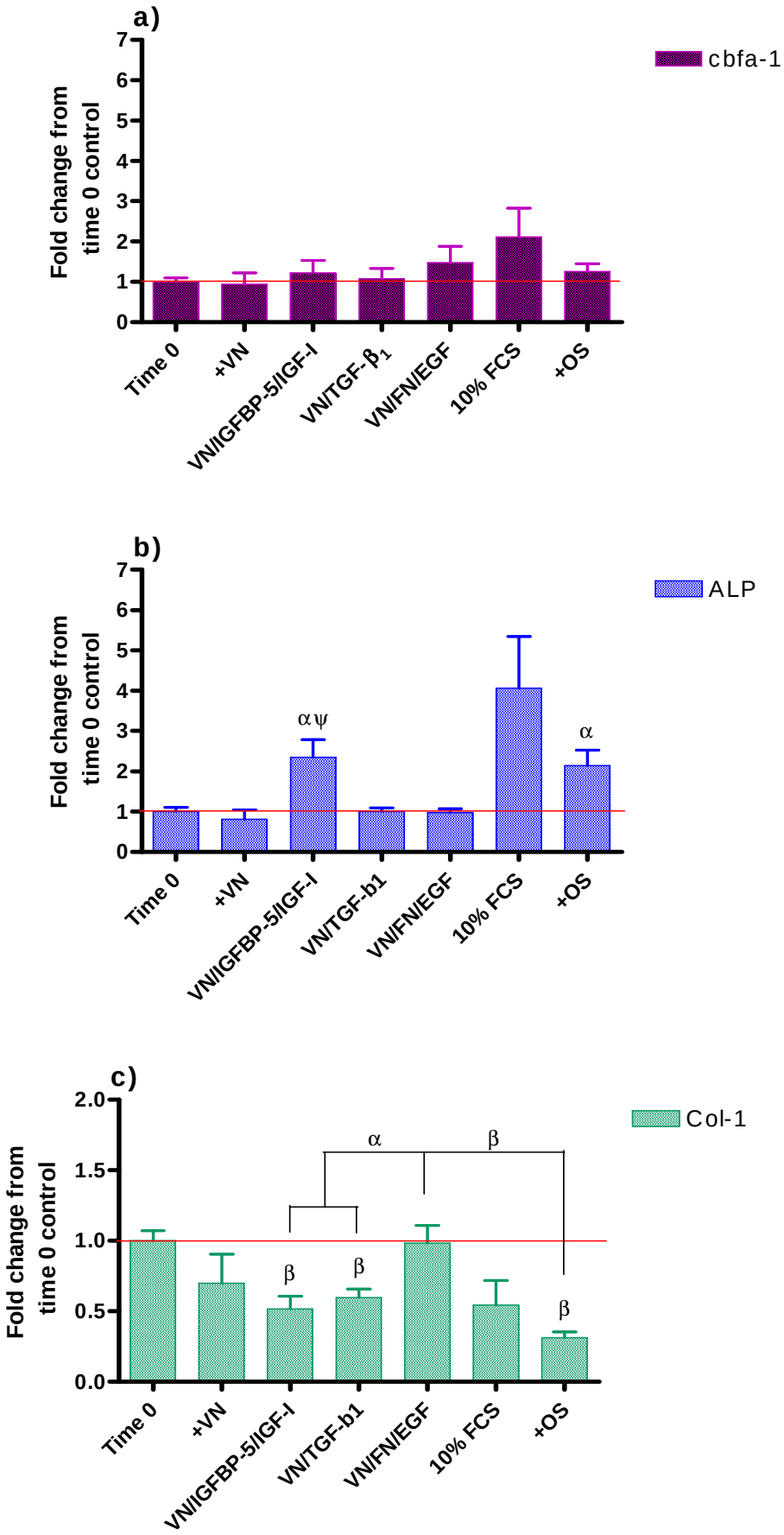

a)
cbfa-1
Fold change from time 0 control
0
1
2
3
4
5
6
7
Time 0
+VN
VN/IGFBP-5/IGF-I
VN/TGF-β1
VN/FN/EGF
10% FCS
+OS
b)
ALP
Fold change from time 0 control
αψ
α
Time 0
+VN
VN/IGFBP-5/IGF-I
VN/TGF-b1
VN/FN/EGF
10% FCS
+OS
c)
Col-1
Fold change from time 0 control
0.0
0.5
1.0
1.5
2.0
α
β
β
β
β
Time 0
+VN
VN/IGFBP-5/IGF-I
VN/TGF-b1
VN/FN/EGF
10% FCS
+OS

Figure 5.8

and VN / TGF-β_1 after 7 days, suggest that either these treatments down regulate Col-I expression over this time or that there is a relatively high transcript number in the SaOS-2 cells at T_0 immediately prior to seeding. Thus these findings suggest that the VN only, VN / IGFBP-5 / IGF-I and VN / TGF-β_1 had no effect on Col-I transcription, and the low transcript number measured in cells exposed to these treatments may be the result of a reduction in transcript number due to the absence of an appropriate stimulus. Interestingly, the VN / FN / EGF treatment seemed to maintain Col-I transcription at basal levels equivalent to that in the T_0 control.

5.4 DISCUSSION

In Chapter 3 I reported that the pre-bound combination of VN, IGFBP-5 and IGF-I can stimulate enhanced cell migration, metabolic activity and cell proliferation in osteoblast-like SaOS-2 cells. Each of these proteins are present in bone matrix and play important roles in bone development and remodelling (Bautista *et al.* 1990; Bautista *et al.* 1991; Seiffert 1996; Kumagai *et al.* 1998; Schvartz *et al.* 1999). Another important constituent of both bone matrix and blood plasma (like VN) is the ~450-500 kDa glycoprotein FN (Yamada and Olden 1978; Yamada and Kennedy 1979; Grzesik and Robey 1994). Like VN, FN also has an RGD integrin binding motif and can therefore facilitate cell adhesion to various culture surfaces (Steele *et al.* 1995; Carvalho *et al.* 1998; Degasne 1999; McFarland *et al.* 1999). Although, it is thought that it is the presence of VN in FCS which facilitates the adhesion of cells to the culture surface in standard *in vitro* tissue culture settings (Underwood and Bennett 1989).

VN molecules attached to tissue culture substrates have an estimated monolayer saturation limit of 0.21 µg/cm^2 (Pitt *et al.* 1989). Similarly, monolayers of FN molecules bound to tissue culture plastic in a 'side on' arrangement (as opposed to 'end on') have a saturation limit of 0.36 µg/cm^2 (Pitt *et al.* 1989). However, interrogation of tissue culture surfaces which have been exposed to high concentrations of VN or FN with monoclonal antibodies, has revealed that these molecules may undergo 'multilayering' (Underwood *et al.* 1993). VN and FN share many desirable properties important for applications in tissue engineering, such as the ability to adhere to a variety of tissue culture and biomaterial surfaces, the ability

to facilitate cell adhesion to those surfaces (Pitt *et al.* 1987; Pitt *et al.* 1989; Degasne 1999; Ku *et al.* 2005), activate integrin mediated signalling pathways (Carvalho *et al.* 1998; Krause *et al.* 2000) and are able to bind IGFBP-5 (Gui and Murphy 2001; Xu *et al.* 2004). Thus, I hypothesised that pre-bound combinations of FN, IGFBP-5 and IGF-I could stimulate similar functional responses in SaOS-2 cells as was observed for VN, IGFBP-5 and IGF-I.

The study reported in this chapter has shown that when FN, IGFBP-5 and IGF-I are pre-bound to polycarbonate Transwell™ membranes, the osteoblast-like cell line SaOS-2 exhibits enhanced cell migration in serum-free conditions compared to either FN alone or similar treatments incorporating VN. In addition, I have shown that FN pre-bound to tissue culture plastic in the presence, or absence, of pre-bound VN dose- dependently influences SaOS-2 cell survival over 72 hr and that the pre-bound combination of FN, IGFBP-5 and IGF-I can enhance SaOS-2 cell survival compared to FN alone, but not compared to VN alone. Specifically, I have demonstrated that SaOS-2 cell migration across 12 µm pore polycarbonate Transwell™ membranes coated with FN is significantly enhanced by approximately 46% compared to membranes exposed to stoichiometrically equivalent amounts of VN, regardless of the presence or absence of additional growth factors (Figure 5.1).

Similar results have been described previously for polymorphonuclear cell migration in response to FN alone and VN alone (Everitt *et al.* 1996). Thus Everitt *et al.* (1996) reported an approximate 50% increase in cell migration on FN coated filters compared to blank or VN-coated filters and that this enhanced response was mediated by $\alpha_5\beta_1$ integrins. I also observed that the relative difference between the various FN based treatments were similar to responses observed for the VN based treatments detailed in chapter 3 and figure 3.1, with the exception that the FN, IGF-I treatment simulated significantly more cell migration than the FN only treatment (Figure 5.1). Significant, however, was the finding that pre-bound combinations of FN, IGFBP-5 and IGF-I could synergistically enhance SaOS-2 cell migration across the Transwell™ membranes, similar to the results obtained for VN coated Transwell™ membranes described in chapter 3. This result contradicts the findings of Xu and co-workers who used a similar migration assay system to the one used in

this study, and recently reported that IGFBP-5 binds to FN and that this interaction negatively regulates the potentiating effect of IGFBP-5 on IGF-I-mediated mouse embryonic cell (MEC) migration (Xu *et al.* 2004). The authors found that IGFBP-5, while bound to FN, could still bind IGF-I in a trimeric complex, similar to the findings of others in respect to IGFBP-5 / IGF-I complex formation with other ECM proteins such as VN, osteopontin and thrombospondin (Nam *et al.* 2000; Nam *et al.* 2002; Kricker *et al.* 2003). While Kricker *et al.* (2003) and Nam *et al.* (2000, 2003) found that complex formation between IGFBP-5 and IGF-I with VN, osteopontin or thrombospondin further stimulated the potentiating effects of IGFBP-5 on IGF-I mediated cell function, Xu and co-workers reported that the presence of FN abolished the potentiating effect of IGFBP-5 on IGF-I mediated MEC migration (Nam *et al.* 2000; Nam *et al.* 2002; Kricker *et al.* 2003; Xu *et al.* 2004). They also demonstrated that this may be due to the expression of IGFBP-5 degrading proteases by MECs in response to exposure to FN. Specifically, they conclude that these enzymes degrade the IGFBP-5 that is bound to FN, thus abolishing its ability to modulate IGF-I mediated effects on cell migration. However, Xu *et al.* 2004 added IGFBP-5 in solution to MECs in the top of Boyden chambers and IGF-I in solution to the bottom well with FN either coated onto the underside of the upper chamber or added in solution together with the MECs and IGFBP-5 with or without protease inhibitors in to the upper chamber (Xu *et al.* 2004). Thus an alternative explanation of their data may be that upon exposure to FN, MECs express IGFBP-5 proteases which degrade IGFBP-5 and / or IGFBP-5 / IGF-I complexes in solution, thus abolishing the ability of IGFBP-5 to efficiently localise IGF-I to the cell surface and positively modulate MEC migration.

In the present study, I pre-bound FN, IGFBP-5 and IGF-I to the lower chamber and underside of Transwell™ membranes prior to the seeding of SaOS-2 cells into the upper chamber in serum-free media. Thus any IGFBP-5 / IGF-I complexes present were bound to either the FN or potentially, non-specifically to the underside of the lower chamber and membrane face and were not in solution. It has been postulated by others that ECM bound IGFBP-5 is protected from proteolytic degradation which facilitates its positive effects on IGF-mediated cell function (Jones *et al.* 1993; Arai *et al.* 1994; Conover 1995). It is concluded that pre-bound combinations of FN, IGFBP-5 and IGF-I enhance cell migration in SaOS-2 cells. I acknowledge that it is

possible that the response observed in the two individual studies is specific to the particular types of cells used and in particular, to differences in the integrins present on the cell surface of these 2 cell types. However, I would hypothesise that application of the migration assay conditions employed by Xu and co-workers, but employing SaOS-2 cells, would yield similar results to those reported by Xu *et al.* 2004 for the MECs. That is, the manner in which the combination of matrix protein and growth factors are presented to the cells is critical to the proper elucidation of the mechanisms underlying cell functions. These findings clearly have further general implications for the use of FN-based ECM / GF coatings on biomaterials. This aspect will be discussed more thoroughly in the next chapter.

Osteoblast cell survival is highly dependent on attachment to a surface, a phenomenon known as anchorage-dependence (Hynes 1987; Ruoslahti and Reed 1994). This regulation of cell survival is mediated in part by integrin receptor initiated intracellular signalling pathways such as the anti-apoptotic FAK, PI 3-kinase, AKT pathway and the pro-apoptotic Bcl-2, caspase activation pathway (Howe *et al.* 1998; Giancotti and Ruoslahti 1999; Grigoriou *et al.* 2005). For example, Zhao and co-workers (2005) demonstrated that the β_3 subunit of unoccupied $\alpha_v\beta_3$ integrins transmitted a "death" signal by association and activation of the pro-apoptotic protease caspase-8 in osteoclasts (Zhao *et al.* 2005). Various integrins are capable of binding to FN (Ruoslahti 1988; Pankov and Yamada 2002) and SaOS-2 cells are known to express both $\alpha_5\beta_1$ and $\alpha_v\beta_3$ integrins (Koistinen *et al.* 1999; Postiglione *et al.* 2003; Kilpadi *et al.* 2004), receptors for FN and VN respectively. In the present study I found that pre-bound FN alone could sustain the SaOS-2 cell population in a dose-dependent manner for 72 hr but could not facilitate cell proliferation over the same time period (Figure 5.2). Maximal SaOS-2 cell survival was achieved by coating the tissue culture surface with FN concentrations of 6-12 µg/mL (Figure 5.2c). Interestingly, this functional response to pre-bound FN correlates well with the observation of Underwood and co-workers (1993) that BHK cell adhesion was maximal at the monolayer saturation limit of FN to tissue culture polystyrene (0.36 µg/cm^2) which correlates to coating the culture surface with an approximate 10 µg/mL of FN solution (Underwood *et al.* 1993; Garcia *et al.* 1999). Thus this suggests that: 1) the survival of 5000 SaOS-2 cells required the FN surface

treatment to be at, or above, the saturation limit for FN on tissue culture polystyrene; and 2) FN coatings that are below the monolayer saturation limit (6-12 µg/mL) cannot sustain the initial number of cells seeded. Whether this is because pre-bound FN at concentrations below the saturation limit does not provide sufficient cell attachment sites to retain all cells or because a rapid reduction in the number of cell attachment sites occurs due to proteolytic degradation of FN molecules, remains unclear.

Since both FN and VN are significant constituents of serum I wished to examine the effect that pre-binding both FN and VN to the tissue culture surface had on cell proliferation. Unexpectedly, I found that the lower concentrations of FN seemed to inhibit SaOS-2 cell survival in the presence of VN, while at the highest concentration of FN, SaOS-2 cell number was significantly increased above that found with VN alone after 72 hr ($p<0.01$). This suggests that the cells had proliferated in response to this particular treatment (Figure 5.3c). This response is reminiscent of a threshold effect whereby high concentrations of cell attachment sites are required to initiate the proliferative response. This hypothesis is supported by the studies which show that both FN and VN can bind to tissue culture plastic in much greater amounts than their respective monolayer saturation limits (0.36 µg/cm^2 and 0.21 µg/cm^2 respectively)(Pitt *et al.* 1987; Pitt *et al.* 1989). However, to achieve this density, FN molecules change orientation from 'side-on' to 'end-on', or undergo multilayering with a concomitant change in conformation. VN molecules tend to multilayer at higher concentrations without apparent changes in conformation (Underwood *et al.* 1993). Therefore it is possible that in the present study more integrin binding sites were available at the highest dose of FN, thus facilitating a proliferative response. Changes in FN conformation in response to differences in culture surface properties, or the presence of type I collagen, can modulate between specific integrin-mediated cell functions, such as proliferation and differentiation of myogenic cells (Garcia *et al.* 1999). Garcia *et al.* (1999) concluded that this effect was due to conformation dependent differences in the level of $\alpha_5\beta_1$ integrin binding to FN. Thus treatment of tissue culture surfaces with multiple matrix proteins can affect the conformation of those proteins and therefore the specific suite of integrins able to bind, thus modulating subsequent cell function. Clearly, further studies are required to

characterise the conformational nature of, in particular, simple combinations of ECM protein coatings on tissue culture polystyrene in order to more fully understand the particular functional consequences of matrix / culture surface interactions and thus desirable surface properties for future biomaterial constructs.

As discussed above, IGFBP-5 has previously been shown to interact with FN and this study and others have shown that the combination of FN, IGFBP-5 and IGF-I can positively and / or negatively modulate cellular responses such as cell migration (Jones *et al.* 1993; Mohan *et al.* 1995; Xu *et al.* 2004). This in turn, has important implications for the design of advanced biomaterial / ECM constructs. Thus, I also wished to examine whether pre-bound IGFBP-5 and IGF-I in the presence of pre-bound FN would have a potentiating effect on cell proliferation, another important possible design feature of advanced tissue engineered constructs. I found that after 72 hr pre-bound FN, IGFBP-5 and IGF-I could sustain significantly higher SaOS-2 cell numbers than FN alone, but was not able to enhance the response of SaOS-2 cells cultured in the presence of pre-bound VN, IGFBP-5 and IGF-I (compare Fig 3.6 and Fig 5.4). In fact, cell number in all serum-free treatments declined after peaking at 48 hr. However the decline in cell number was smaller in the presence of FN, IGFBP-5 and IGF-I compared to the other FN treatments. This result, together with the results detailed in chapter 3, suggest that either: 1) SaOS-2 cells are interacting with FN and VN via different mechanisms; or 2) there are fewer binding sites available for engagement of IGFBP-5 / IGF-I complexes and thus limited receptor engagement and mitogenic signaling. Further studies are required to determine the amount of IGFBP-5 and IGF-I that is capable of being retained by both FN and VN, or combinations thereof, immobilised on tissue culture plastic. As pointed out above, determination of how much, if any, IGFBP / IGF-I binds to tissue culture polystyrene from serum under standard culture conditions would be of significant interest.

In a related study in which I examined responses to solution phase FN and VN, with or without EGF or bFGF (known to bind to VN and induce functional responses in other cell lines (Schoppet *et al.* 2002; Hollier *et al.* 2005), preliminary morphological data suggested that the combination of VN, FN and EGF could sustain SaOS-2 cell viability for extended periods of time (12 days)(Figure 5.5d). I therefore decided to

include this treatment in a pre-bound format in subsequent experiments evaluating the effect of select treatments on morphology and osteoblast marker expression in SaOS-2 cells. Other specific serum-free treatments which had yielded interesting or intriguing results throughout this study were selected for evaluation of their effect on SaOS-2 cell morphology and select osteoblast marker expression. These treatments were: VN alone (as a base line treatment); VN / IGFBP-5 / IGF-I (due to the enhanced proliferation and metabolic activity it induced in SaOS-2 cells and reported in chapter 3); VN / TGF-β_1 (due to the enhanced response it induced in hMSC metabolic activity, as detailed in chapter 4, and for the reported effects both proteins have on osteogenic differentiation (Salasznyk *et al.* 2004a; Salasznyk *et al.* 2004b); and VN / FN / EGF (for the intriguing effect this combination had on SaOS-2 cell morphology and apparent survival, as detailed above).

Osteoblast *in vitro* morphology typically changes during osteogenic differentiation from a spread / polygonal, or fibroblastic morphology, to a compacted and cobblestone / cuboidal morphology (Aubin 1998). I therefore compared the morphology of SaOS-2 cells exposed to pre-bound VN alone; VN / IGFBP-5 / IGF-I; VN / TGF-β_1; or VN / FN / EGF with SaOS-2 cells cultured in 10% FCS or 10% FCS supplemented with osteogenic factors (OS media) over 7 days. The osteogenic supplements were utilised to drive the differentiation process and marker expression. SaOS-2 cells cultured in this environment represent a model of rapidly differentiating cells whereas, SaOS-2 cells cultured in the presence of 10% FCS undergo terminal differentiation over a longer time period and as such represent a model of more slowly differentiating cells (data not shown). After 7 days of culture SaOS-2 cells exposed to pre-bound VN, IGFBP-5 and IGF-I adopted a more cobblestone / cuboidal morphology similar to that observed for SaOS-2 cells cultured in 10% FCS or OS media (Figure 5.6b). Dexamethasone, a glucocorticoid constituent of OS media is known to induce osteogenic differentiation in hMSCs including a morphological transformation from fibroblastic to cuboidal (Cheng *et al.* 1994). Thus, the morphology of SaOS-2 cells exposed to VN / IGFBP-5 / IGF-I, 10% FCS and OS media is consistent with the process of osteoblast differentiation. The key difference, however, between these results is that cells exposed to VN / IGFBP-5 / IGF-I did not proliferate to the same extent as cells exposed to 10% FCS and they began to adopt a more cobblestone appearance while sub-confluent. This

suggests, that VN, IGFBP-5 and IGF-I may facilitate SaOS-2 cell differentiation processes and that other factors, which are present in FCS, are required to drive the proliferative response.

These conclusions were further supported by the finding that VN / IGFBP-5 / IGF-I also facilitated the increase (Figure 5.7b) and maintenance of ALP gene expression over 7 days compared to the T_0 control (unseeded SaOS-2 cells) whereas other serum-free, VN containing, treatments did not sustain the initial increase in ALP expression above the T_0 control (Figure 5.8b). In a parallel study also performed in our laboratory, the VN / IGFBP-5 / IGF-I treatment induced a consistent increase in ALP activity over 14 days (Figure 5.9).

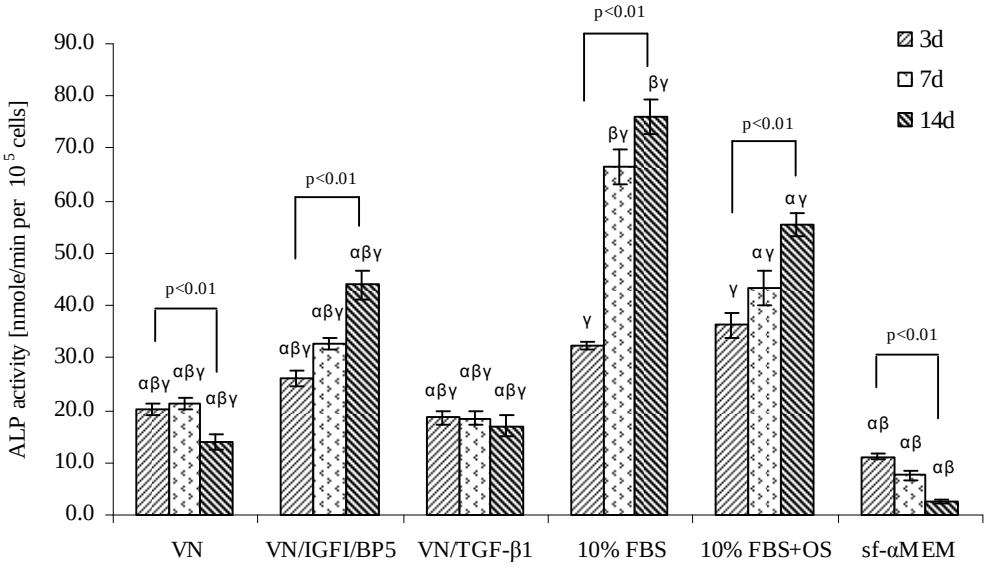


Figure 5.9. Effect of VN, alone or in combination with IGFBP-5 / IGF-I or TGF-β_1, on ALP activity per 10^5 SaOS-2 cells. Alkaline phosphatase activity is expressed as nmol of *p*-nitrophenol produced per minute per 10^5 cells ± SEM. Statistical significance was determined using Student's t-test ($p<0.05$ or $p<0.01$). Significant difference ($p<0.05$) to control groups at the same time point are indicated by α (10% FCS), β (10%+OS), or γ (sf-αMEM). (Reproduced with permission of K, Shrobback, Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology).

Since SaOS-2 cells are known to express high levels of ALP (Murray *et al.* 1987) it is possible that the initial increase in ALP transcription and the accumulation of ALP activity induced by VN, IGFBP-5 and IGF-I may represent facilitation of a more 'normal' phenotype rather than a differentiation response. The apparent

disengagement of ALP gene transcription (Figure 5.7b and 5.8b) and enzymatic activity (Figure 5.9) over extended time may be an artefact of the ALP bioassay. The ALP assay data is a measure of the accumulated enzyme in the system, whereas the real-time PCR data more closely reflects the status of gene transcription at the time of RNA extraction (a "snap-shot"). Alternatively, these data may also reflect discordance between transcriptional and post translational regulatory mechanisms. Indeed, expression of the ALP gene has been shown to be regulated very early post transcripionally by the presence of destabilising intronic sequences (Kiledjian and Kadesch 1991). Furthermore, its functional activity is regulated by inorganic phosphate sources (Farley *et al.* 1994; Orimo and Shimada 2006), while regulation of its release by SaOS-2 cells is Ca^{2+} and growth factor dependent (Anh *et al.* 1998). Clearly further studies are required to fully elucidate the mechanisms of action of VN / IGFBP-5 / IGF-I on ALP expression in SaOS-2 cells and the effects of the addition of the components in solution following the initiation of cellular responses on the pre-bound format. From a tissue engineering point of view this would be important because it is conceivable that for some *in vitro* tissue engineered constructs, media changes would be required and thus knowledge of how cellular mechanisms are modulated by the provision of various signals in solution will be an important design consideration for such constructs.

The parallel study by Shrobback *et al* mentioned above, also revealed that SaOS-2 cells exposed to either VN alone; VN / IGFBP-5 / IGF-I; or VN / TGF-β_1 incorporated increasing levels of [^{3}H]-Proline, as a percentage of total protein synthesis over time, compared to SaOS-2 cells exposed to 10% FCS alone (Figure 5.10). [^{3}H]-Proline incorporation is a commonly used functional bio-assay for *de novo* collagen synthesis in bone cells (McCarthy *et al.* 1989; Layman and Ardoin 1998) and hence these data seem to suggest either that the presence of VN could facilitate the incorporation of L-proline into accumulated protein or, that the presence of unknown / unidentified factors within FCS suppress the increased incorporation of L-proline. Indeed, the elevated levels of target gene expression (cbfa-1, and ALP) in the 10% FCS treatment compared to the other treatments may reflect the combined effect of high metabolic activity induced by factors within FCS, coupled with a contact-mediated differentiation response.

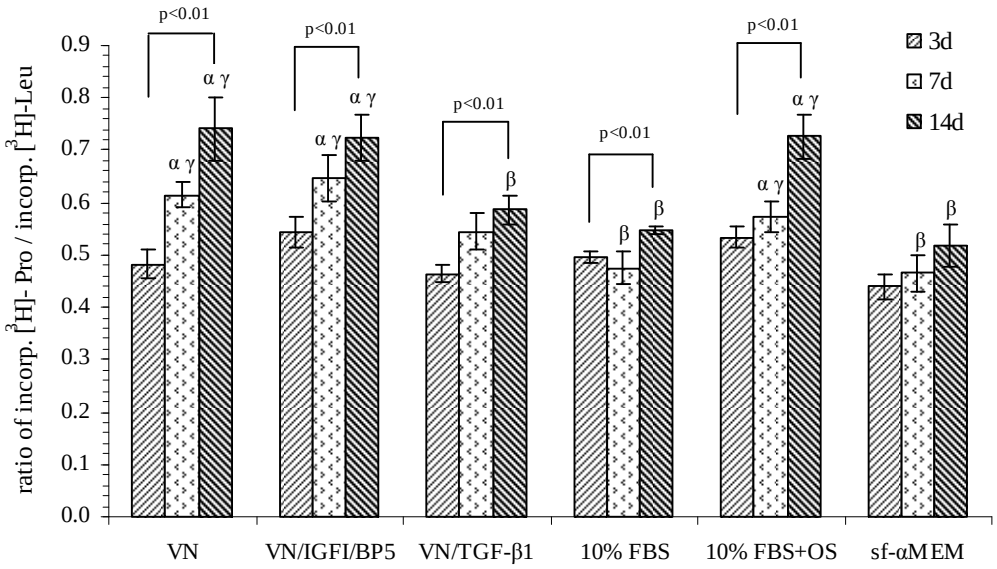


Figure 5.10. Effect of VN, alone or in combination with IGFBP-5 / IGF-I or TGF-β_1, on the ratio of [^{3}H]-Pro and [^{3}H]-Leu in newly synthesised proteins in SaOS-2 cells. Results are expressed as the mean ratio of [^{3}H]-Pro to [^{3}H]-Leu in newly synthesised protein ± SEM. Statistical significance was determined using Student's t-test (p<0.05 or p<0.01). Significant difference (p<0.05) to control groups at the same time point are indicated by α (10% FCS), β (10%+OS) or γ (sf-αMEM). (Reproduced with permission of K Shrobback, Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology).

While VN has been associated with induction of osteogenic differentiation processes in hMSCs, such as type I collagen expression (Salasznyk *et al.* 2004b), others have observed that over-expression of $\alpha_v\beta_3$ integrins promotes cell proliferation with decreased expression of a number of key markers of osteogenic differentiation (Cheng *et al.* 2001). However, integrin (including $\alpha_v\beta_3$ integrin) interaction with the ECM is essential to facilitate osteoblast-mediated matrix mineralisation (Schneider *et al.* 2001). Indeed, the expression of the osteoblast differentiation marker osteopontin (OPN) has been shown to be up-regulated in cells over expressing $\alpha_v\beta_3$ integrins (Cheng *et al.* 2001) while others report that OPN gene expression is not altered by osteoblast attachment to the $\alpha_v\beta_3$ integrin ligands OPN or VN (Carvalho *et al.* 1998). Similarly, IGF-I has been shown to increase L-proline uptake and type I collagen synthesis in SaOS-2 cells (Kudo *et al.* 1996) and has been shown to be involved in the suppression of apoptosis and maintenance of the differentiated phenotype of more mature osteoblasts (Walsh *et al.* 2003). IGF-I is further reported

to mediate survival and proliferative responses in SaOS-2 cells via PI3-Kinase and p42 / 44 MAPK pathways (Grey *et al.* 2003) and similarly IGFBP-5 is associated with both osteoblast mitogenesis and bone formation (for a recent review of the role of IGFBP-5 in bone the reader is referred to (Govoni *et al.* 2005)). Thus the overall combined effects of VN, IGFBP-5 and IGF-I in a pre-bound format in serum-free media on osteoblast function remain unclear and warrant further investigation.

One of the most intriguing findings in the present study was that within 48 hr SaOS-2 cell cultures exposed to VN / FN / EGF were confluent and adopted an elongated or fibroblast-like morphology and maintained this until day 7 (Figure 5.6a.4 and 5.6b.4). Apart from an initial increase in ALP expression after 48 hr, the cbfa-1, ALP and Col-I transcript levels had returned to (ALP), or were maintained (cbfa-1 and Col-I), at levels equivalent to T_0 after 7 days (Figure 5.7 and 5.8). Taken together, these data suggest that the VN, FN and EGF treatment induced a quiescent state on the cells. Hence, while transcription levels of cbfa-1, ALP and Col-1 had returned to T_0 levels, the cultures displayed few mitotic indices compared to cultures exposed to FCS (Figure 5.6a and b). Furthermore, as mentioned above, this treatment could also sustain the cells in a similar morphological state for up to 12 days of culture (Figure 5.5). This conclusion is supported by studies which have shown that EGF, via the EGF receptor (EGFR), inhibits osteogenic differentiation processes such as collagen synthesis and ALP activity (Kumegawa *et al.* 1983; Chien *et al.* 2000). For example, transcription of the osteoblast marker osteocalcin (OC) has been shown to be negatively regulated by Fos and Jun heterodimers binding to the AP-1 site within the vitamin D response element the of the OC gene promoter, inhibiting both vitamin D mediated and basal OC gene transcription (Owen *et al.* 1990). EGF is known to up-regulate Fos and Jun production (Marks *et al.* 1996; Wan *et al.* 2001), thus providing a mechanism for EGF- and EGFR-mediated down regulation of osteoblast differentiation and marker expression (Chien *et al.* 2000). Interestingly, FN has been associated with the development of the osteoblast phenotype, especially on a pre-bound type I collagen culture surface (Stephansson *et al.* 2002). In addition, the $\alpha_5\beta_1$ integrin / FN interaction was found to be critical for rat calvarial osteoblast differentiation *in vitro*, and perturbation of this interaction by function blocking antibodies down-regulated ALP and OC gene expression (Moursi *et al.* 1996;

Moursi *et al.* 1997). Although the same authors demonstrated that FN can act as a survival factor for more mature osteoblasts (Globus *et al.* 1998), the mechanism(s) controlling, or contributing to the apparent quiescence of SaOS-2 cell exposed to VN / FN / EGF remain unclear. It is important to examine if the apparent quiescence is reversible, and if the cells could be further induced to express a more differentiated phenotype by, for example, removal of the EGF and replacement with a known osteogenic factor such as bone morphogenetic protein 2 or 7. This level of control over cell phenotype in serum-free culture conditions will have application in the field of bone tissue engineering for the development of cell / biomaterial constructs.

In conclusion, the data presented in this chapter suggest that FN together with IGFBP-5 and IGF-I (in serum-free conditions) can stimulate enhanced SaOS-2 cell migration, but not proliferation, over and above the levels attained for VN / IGFBP-5 / IGF-I reported in chapter 3. Furthermore, pre-bound FN alone in serum-free conditions could facilitate SaOS-2 cell survival over 72 hr in a dose-dependent manner, while at high concentrations and in the presence of pre-bound VN, SaOS-2 cell numbers are increased. In addition, I have also shown that VN / IGFBP-5 / IGF-I in serum-free conditions can facilitate an increase in ALP expression and activity and adoption of a more differentiated morphology by SaOS-2 cells. Furthermore, the VN / FN / EGF serum-free treatment seemed to induce quiescence in SaOS-2 cells while maintaining cbfa-1, ALP and Col-1 mRNA levels similar to those present at the time of seeding. While it is obvious that further investigations into the mechanisms underlying these results are required, it is clear that these treatments represent a basic starting platform of a defined ECM / growth factor, serum-free culture system that might hold potential in controlling cell phenotype for tissue engineering applications.

CHAPTER 6:

GENERAL DISCUSSION

6.1 DISCUSSION

The field of bone tissue engineering has continued to expand over the 19 years when the formal definition of the term 'tissue engineering' was first developed at a meeting of representatives of the biotechnology and engineering community in Lake Tahoe, California in February 1988. The broad definition of the term 'tissue engineering' is the use of living cells, together with either natural or synthetic extra cellular components *in vitro*, for the development of implantable parts or devices for the restoration or replacement of tissue or organ function (Patrick *et al.* 1998). There have been several seminal advances critical for the advancement of the field of tissue engineering, one of which was the discovery and isolation of so-called mesenchymal stem cells from bone marrow (Friedenstein *et al.* 1970; Friedenstein *et al.* 1987; Caplan 1991; Friedenstein *et al.* 1992). This discovery has provided a source of cells which have a proven ability to differentiate into multiple lineages and affect tissue repair in both animal models and humans (Bruder *et al.* 1998a; Bruder *et al.* 1998b; Pittenger 1999; Caplan and Bruder 2001; Grayson *et al.* 2004; Otto and Rao 2004; Warren *et al.* 2004). Other more recent advances include the discovery that members of the IGF family of growth factors could interact directly with the ECM protein VN and that this enhances functional responses in porcine smooth muscle cells and MCF-7 breast cancer cells (Upton *et al.* 1999; Nam *et al.* 2002; Kricker *et al.* 2003). Inspired by these early findings members of our research team quickly realised that the adhesive properties of VN, coupled with the ability to associate with a growth factor, could be used as a delivery mechanism to immobilise the growth factor at the culture surface where it could be accessed to enhance various cellular responses. In particular, it was postulated that this may more accurately reflect the environment that cells encounter *in vivo*. Numerous studies have since demonstrated that select pre-bound combinations of VN ± IGF-II and VN ± the 6 IGFBPs ± IGF-I are able to mediate various functional responses in MCF-7 cells and HaCAT keratinocytes (Noble *et al.* 2003; Hyde *et al.* 2004). Since these initial observations, other growth factors have also been shown to also interact with VN; these include EGF, bFGF and TGF-β_1 (Schoppet *et al.* 2002). In addition, when these growth factors are presented to cells *in vitro*, in association with VN, cellular responses are greater than responses to the

individual components (Schoppet *et al.* 2002; Hollier *et al.* 2005), suggesting interactions between distinct receptor induced signaling pathways.

Vitronectin and members of the IGF family of growth factors and TGF-β_1 are important components of bone tissue (Bautista *et al.* 1990; Bautista *et al.* 1991; Seiffert 1996) and VN is the principal protein to adsorb to tissue culture plastic from serum containing media (Bale *et al.* 1989; Pitt *et al.* 1989; Steele *et al.* 1995). The broad aim of this PhD project was to characterise the functional responses of human osteoblasts and human mesenchymal stem cells to pre-bound combinations of VN, IGFBP-5 and IGF-I under serum-free conditions, with a view to expanding the study to include other biological factors important to musculoskeletal repair such as FN, TGF-β_1 and EGF. Therefore, this thesis has principally focussed on functional analyses such as cell migration, metabolic activity, proliferation, total protein assays and morphological analysis.

Overall, the hypothesis proposed in chapter 1 that: 'Specific combinations of the ECM protein VN and members of the IGF family of growth factors, such as IGFBP-3 or -5 and IGF-I can support cell attachment, migration, proliferation and / or differentiation of human osteoblast like cells (SaOS-2) and hMSCs in culture in the absence of animal derived culture media additives is supported by a number of key findings reported in this thesis. Experimental observations reported in chapter 3 specifically demonstrates SaOS-2 cell migration is significantly enhanced in the presence of VN together with IGFBP-5 and IGF-I. In addition, VN / IGFBP-5 / IGF-I enhanced SaOS-2 cell metabolic activity after 48 hr and manifests as enhanced proliferation after 72 hr. These findings are important because osteoblast adhesion to, migration and colonisation of 3 dimensional biomaterial scaffolds are desirable in bone tissue engineering contexts (Anselme 2000). Thus, these findings indicate that pre-bound VN / IGFBP-5 / IGF-I may have potential as a bioactive coating for tissue engineered biomaterial constructs. This conclusion is further supported by the fact that (as mentioned above) VN is a matrix component of bone (Seiffert 1996) and IGFBP-5 and IGF-I are highly expressed in bone tissue (Bautista *et al.* 1990; Bautista *et al.* 1991). Indeed, others have investigated VN for its value as a potential bioactive coating for 3 dimensional biomaterial constructs for tissue

engineering applications (Fabrizius-Homan and Cooper 1991; Degasne 1999; Webster *et al.* 2001; Lacouture *et al.* 2002; Ku *et al.* 2005; Schleicher *et al.* 2005).

A pertinent observation also reported in chapter 3 was that metabolic activity assayed after 48 hr permitted the increased SaOS-2 cell numbers measured after 72 hr of culture on VN / IGFBP-5 / IGF-I. In contrast, other treatments were unable to sustain SaOS-2 metabolic activity resulting in fewer cells in the same period of culture. Importantly, since there is a clear discrepancy between the 48 hr metabolic activity data and cell number observed at the same time point, these data clearly demonstrate that the use of assays based on metabolic activity are far from ideal measures of proliferation. Significantly however, these data also show that by understanding the limitations of such assays one may apply such data to build a more complete picture of the functional response to given treatments / surfaces etc. This is especially true in relation to *bona fide* cell proliferation which would logically require an increase in cellular metabolic activity prior to an increase in cell number. Future studies should incorporate functional analyses of metabolic activity, total protein synthesis and cell number over time as a matter of course in order to construct a more complete picture of the functional effects of these and other combinations of proteins within *in vitro* settings. Moreover, such approaches would avoid the common misinterpretation or over-interpretation of experimental data.

A prolific body of evidence has been generated on the isolation, characterisation, propagation and therapeutic potential of so-called human mesenchymal stem cells (hMSCs) (Haynesworth *et al.* 1992; Bruder *et al.* 1994; Caplan 1994; Wakitani *et al.* 1994; Jaiswal *et al.* 1997; Caplan *et al.* 1998; Johnstone *et al.* 1998; Solchaga *et al.* 2001). Indeed, hMSCs cultured *in vitro* and seeded onto various types of biomaterial scaffold materials have a demonstrated ability to repair large segmental bone defects in animal models and by implication suggest that this technology has an equivalent potential in human patients (Bruder *et al.* 1994; Bruder *et al.* 1998a; Bruder *et al.* 1998b; Turgeman *et al.* 2001). A major obstacle to the clinical use of *ex vivo* expanded hMSCs is that current culture methods require the use of animal-derived media supplements such as foetal calf serum (FCS). Regulatory authorities such as the Food and Drug

Administration (FDA) in the USA or the Therapeutic Goods Administration (TGA) in Australia restrict the use of cell based therapeutics which have come into contact with animal derived substances due to the risk of pathological transfer of disease to patients (Federal Register). Bovine Spongiform Encephalitis (BSE) commonly known as 'mad cow disease' is an example of a pathogen which may be transferred to human recipients through cell based therapeutics exposed to contaminated FCS. Other animal derived media supplements such as anterior pituitary extract are also undesirable for therapeutic applications due to the "un-defined" nature of these products which often exhibit significant batch to batch variation. This is the primary reason behind the common practice of batch testing of specific lots / batches of FCS for optimal cell culture performance (Lennon *et al.* 1996). Thus, a key research focus of our team is to examine the efficacy of using various combinations of matrix proteins and growth factors to replace animal derived media supplements for the culture of therapeutically important cell and tissue grafts. Indeed our research team has demonstrated that primary human keratinocytes derived from adult skin can be established and expanded in serum-free conditions using combinations of VN, IGFBP-5, IGF-I and EGF (Dawson *et al.* 2006). Additionally, serum-free methodologies for the culture of primary limbal epithelial cells for treatment of a range of serious eye injuries has also been achieved (Ainscough *et al.* 2006). However, both of these epithelial cell types still required the use of a mouse feeder layer to provide as yet unknown components of the *in vitro* environment. No such feeder layer is required for the establishment and serial propagation of hMSCs. Furthermore, the argument for developing a serum-free medium which incorporates VN is convincing since VN is the principal protein which is adsorbed on to tissue culture plastic from serum (McFarland *et al*. 1999) in standard culture conditions. Thus, I would hypothesise that VN should be a principal component in any rational serum replacement for the *in vitro* culture of cells, given that most experimental data derived from *in vitro* studies have included exposure of cells to serum.

The difference in complexity between media containing 10% FCS and media containing VN, an IGFBP and IGF-I is clearly illustrated in figure 6.1.

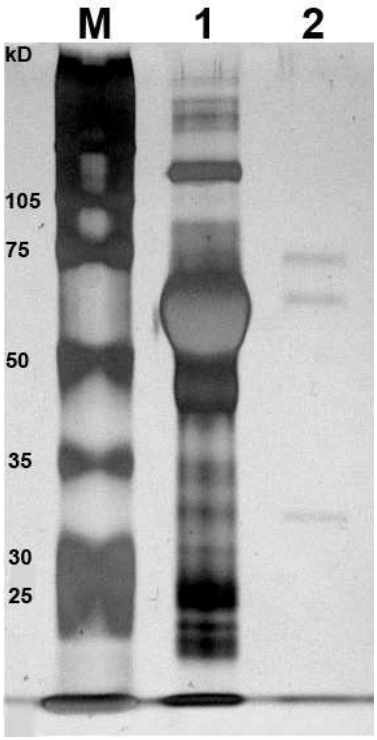


Figure 6.1 Comparison of protein from media with 10% FCS and media with VN, IGFBP and IGF-I
Proteins within DMEM culture media containing 10 % FCS (0.1μL) (lane 1) and media containing VN (75 kDa and 68 kDA), IGFBP-3 (32 kDa) and IGF-I (7.5 kDa not clearly identified on gel) (10μL) (lane 2) were separated by 10% SDS-PAGE under non reducing conditions and subsequently silver stained. (MW marker: lane M). (Reproduced with permission of L Cormack, Tissue Repair and Regeneration Program, Institute of Health and Biomedical Innovation, Queensland University of Technology).

Thus, the ability of the VN, IGFBP-5 and IGF-I formulation to support functional responses in hMSCs was examined. Subsequently, the second aim of this project sought to characterise the initial functional response of hMSCs to various combinations of VN, IGFBP-3 or -5 and IGF-I, or VN and TGF-β_1 in serum-free conditions.

Several novel findings were made during the studies, reported in chapter 4. Initially, I attempted to establish bone marrow-derived fibroblastic cultures directly from the mononuclear cell fraction of whole bone marrow obtained from patients presenting for total joint replacement surgery, using pre-bound combinations of VN and IGFBP-3 or -5 and IGF-I, or VN and TGF-β_1. However, this approach failed to support adequate

fibroblastic cell attachment. Whereas, leaving the unbound fraction of VN and growth factors in solution did support fibroblastic cell attachment and maintenance over 3-4 days of culture but unfortunately this approach did not sustain the cultures beyond this time point. To the best of my knowledge this is the only study which has described the discreet attachment of fibroblast like cells from the mononuclear cell fraction of whole bone marrow, rather than forming in colonies. It is, however, unclear whether or not these cells were hMSCs. Further studies are clearly required to test the colony formation capabilities and multi-lineage potential of these cells.

Based on the above results I decided to characterise the functional response of hMSCs which had been established using classical methods and expanded in culture. Thus, I also reported in chapter 4 that solution phase VN in combination with TGF-β_1, significantly enhanced hMSC metabolic activity in a dose-dependent manner, with responses being greater than those measured after 72 hr in the presence of 10 % FCS. This result indicated that TGF-β_1: 1) has a direct stimulatory effect on hMSC metabolic activity via its down stream signalling effectors; or 2) stimulates metabolic activity indirectly by inducing the expression of other mitogens which act in an autocrine / paracrine fashion. This latter hypothesis is supported by the findings of Kveiborg and co-workers who showed that TGF-β_1 induces the expression of IGFBP-3 and IGF-I in hMSCs (Kveiborg *et al.* 2001). This possibility is intriguing because the presence of exogenous IGFBP-3 and IGF-I did not result in an equivalent response to TGF-β_1 at either concentration of VN tested (52 ng/well or 174 ng/well). However, in the study reported herein an *E.coli* derived IGFBP-3 mutant (N109D) which is non-glycosylated (~35 kDa) was utilised, whereas the 2 isoforms of IGFBP-3 produced by hMSCs exposed to TGF-β_1 were partially (38 kDa) or fully glycosylated (42 kDa) isoforms (Kveiborg *et al.* 2002). The mechanisms underlying the variable response to VN, IGFBP-3 or-5 and IGF-I compared to VN and TGF-β_1 remain unclear; however, it is possible that the expression level and / or post translational modifications of TGF-β_1-induced IGFBP-3 and IGF-I may be important and further studies are required to ascertain if this is indeed the case.

Interestingly, VN together with TGF-β_1, has been reported to increase DNA synthesis in human lung fibroblasts (hLF) via a mechanism which involves the interaction of the TGF-β type II receptor (TGFβIIR) and $\alpha_v\beta_3$ integrins (Scaffidi *et al.* 2004). In this situation, ligand occupancy induced co-localisation and immuno-complex formation of both the $\alpha_v\beta_3$ integrin and the TGFβIIR, increased expression of the $\alpha_v\beta_3$ integrins on the cell surface and synergistically enhanced the hLF proliferative response via up regulation of cyclin D1 (Scaffidi *et al.* 2004). hMSCs are known to express $\alpha_v\beta_3$ integrins (Conget and Minguell 1999) and are responsive to TGF-β_1 (Kveiborg *et al.* 2001). Taken together these mechanistic data support my functional findings that VN and TGF-β_1 synergistically stimulate hMSC metabolic activity and this response is further enhanced with increasing the VN concentration. Moreover, this may be due to an increase in cell surface $\alpha_v\beta_3$ integrins mediating the response to the increase in VN, perhaps in combination with endogenously expressed IGFBP-3 and IGF-I which acts in an autocrine / paracrine manner, resulting in the observed synergistic response. Obviously, the specific mechanisms underpinning this response remain to be elucidated and further investigation of the underlying mechanism(s) is warranted.

In contrast to the metabolic activity data detailed in chapter 4, which revealed a general decrease in metabolic activity between 24 hr and 48 hr assays of total protein revealed no such decrease, suggesting that the observed reduction in WST-1 metabolism was probably not the result of decreased cell numbers. However the increase in metabolic activity between 48 hr and 72 hr does not necessarily indicate an increase in cell number but may instead indicate an increase in extra-cellular protein production. Generally, VN, IGFBP-3 or -5 and IGF-I, or VN and TGF-β_1, facilitated an increase in total protein equivalent to that of hMSCs exposed to 10% FCS over 72 hr. IGF-I has been positively (Jia and Heersche 2000) and negatively (Walsh *et al.* 2003) associated with osteoprogenitor cell proliferation. In addition, others have shown that IGFBP-5 enhances IGF-I mediated mitogenesis in osteoblasts (Andress and Birnbaum 1992), while the growth of porcine smooth muscle cells cultured in the presence of the $\alpha_v\beta_3$ integrin ligand, osteopontin, and IGF-I was found to be enhanced to levels similar to that of cells cultured in 10% FCS by the addition of IGFBP-5 (Nam *et al.* 2000). In further

support of my own observations, Nam and co-workers found that the potentiating effect of IGFBP-5 on IGF-I-mediated DNA synthesis was dependent on the binding interaction of IGFBP-5 to VN (Nam *et al.* 2002). Overall, these data tend to support the results presented in this thesis and related reports which have found that complexes composed of select IGFBPs together with IGF-I, including IGFBP-3 and -5, bound to VN can enhance the functional responses of various cell types (Kricker *et al.* 2003; Hyde *et al.* 2004; Hollier *et al.* 2005; Schleicher *et al.* 2005; Ainscough *et al.* 2006; Dawson *et al.* 2006).

I also reported in chapter 4 that the presence of IGF-I or TGF-β_1 and a relatively low concentration of VN, induced the aggregation of cells in the cultures, whereas a relatively high concentration of VN attenuated this aggregation phenomenon. Furthermore, total protein data suggests that the aggregation effect was not due to a decrease in cell number, since in the presence of the lower concentration of VN, hMSC total protein continued to increase over time, as did cell aggregation over time. If the aggregation effect was due to a reduction in cell number I would have expected to observe a decrease in total protein content over time or at least a plateau in protein accumulation.

Studies performed using the mouse osteoblast cell line (MC3T3-E1 cells) exposed to TGF-β_1 have described similar morphological changes, and reported that in the presence of pre-bound type I collagen these changes were abrogated (Hurley *et al.* 1994; Karsdal *et al.* 2001). Moreover TGF-β_1 was found to increase activation of the ERK1/2 and p38 MAP kinase signalling pathways; however, only the p38 MAP kinase pathway was implicated in the morphological changes (Karsdal *et al.* 2001). Interestingly, TGF-β_1 is known to induce plasminogen activator inhibitor (PAI-1) expression (Boehm *et al.* 1999; Kutz *et al.* 2001) which can mediate cell detachment from VN by initiating endocytosis of uPA-uPAR-α_v integrin complexes. Indeed, PAI-I mediated CHO cell detachment can be drastically reduced by over-expression of $\alpha_v\beta_3$ integrins on the cell surface (Czekay *et al.* 2003). In addition, Tram and co-workers have previously described a similar response to parathyroid hormone in osteoblasts (Tram *et al.* 1993),

thus supporting the observation that higher concentrations of VN can attenuate the aggregation response in hMSCs. This is the first study to describe this type of response in either hMSCs or to IGF-I, and to report that the response can be dose-dependently attenuated by VN. However, the specific mechanisms of the observed aggregation response to both TGF-β_1 and IGF-I in hMSCs remains to be elucidated.

My initial thoughts regarding the growth factor mediated hMSC aggregation phenomenon was that this may have been due to secreted protease activity, indeed pro-MMP-2 and pro-MMP-9 was found to be present in the conditioned media of hMSCs (including hMSCs cultured in serum-free media alone) regardless of treatment. This indicated that 1) hMSCs constitutively express MMP-2 and MMP-9 *in vitro* and 2) none of the treatments had any affect on the functional expression of MMP-2 or MMP-9 by hMSCs. Thus, there was no correlation with the presence of either of these MMPs in the hMSC conditioned media and the aggregation affects of either IGF-I or TGF-β_1. In addition, it appears that the constitutive expression of MMP-2 is relatively common in a number of cell types (Sternlicht and Werb 2001). However, the IGFBPs used in this study are known substrates for each of these MMPs (Fowlkes *et al.* 1995; Sternlicht and Werb 2001) and I also reported in chapter 4 that as yet unidentified factors within the hMSC conditioned media could degrade purified IGFBP-5. Again the data is supported by the findings of others which showed MMP-2 and MMP-13 are present in the conditioned media of MC3T3 osteoblast cells and that the expression of MMP-13, but not MMP-2, had been induced by TGF-β_1 (Karsdal *et al.* 2001). Interestingly, Ray and Stetler-Stevenson (1995) showed that attachment and spreading of melanoma cells on VN could be restored by over expression of the MMP-2 inhibitor tissue inhibitor of metalloproteinase-2 (TIMP-2), despite showing that the VN substrate was not degraded by activated MMP-2 (Ray and Stetler-Stevenson 1995). The expression of, or putative role of, secreted MMP inhibitors on the functional response of hMSCs to VN ± IGFBP-3 or -5 and IGF-I, or VN and TGF-β_1 was not investigated during this study. However, the answers to this and other unknowns, such as the fate of the exogenously added proteins, are vital to understand the relationship between such therapeutically important cells and artificial environments, especially from a tissue engineering context.

In summary, the findings presented in chapter 4 suggest that VN is a valuable matrix component for the initial stimulus of hMSCs metabolic activity and protein sysnthesis in serum-free culture. Moreover, the addition of growth factors such as TGF-β_1 can enhance the response, although culture integrity may depend on the concentration of VN or some other matrix protein. In addition, further investigation is required to determine the effect of endogenously expressed proteases on the function of added matrix components, growth factors or other constituents, as well as the specific mechanisms of action of these exogenous factors on cell function.

Like VN, the matrix protein FN is a major matrix component of blood plasma (Yamada and Kennedy 1979), is an important matrix component within bone tissue (Grzesik and Robey 1994; Moursi *et al.* 1996), facilitates cell adhesion to various surfaces via integrin receptors (Moursi *et al.* 1997; Carvalho *et al.* 1998; Danen and Yamada 2001; Danen *et al.* 2002; Stephansson *et al.* 2002) and has been shown to bind IGFBP-3 and -5 (Gui and Murphy 2001). In addition, the binding interaction between FN and IGFBP-5 has been shown to modulate IGF-I mediated mouse embryonic cell migration (Xu *et al.* 2004). For these reasons, and my observations that VN, IGFBP-5 and IGF-I could modulate enhanced migration and proliferative responses in SaOS-2 cells (as reported in chapter 3), I hypothesised that FN / IGFBP-5 / IGF-I may support similar activities in SaOS-2 cells. In chapter 5, I report that FN / IGFBP-5 / IGF-I synergistically enhanced SaOS-2 cell migration through 12 µm pore Transwell™ membranes. In addition, the trend between the various combinations of FN ± IGFBP-5 ± IGF-I was similar to that reported for treatments which incorporated VN, although the level of the response was on average 46% greater in treatments incorporating FN compared to the same treatment using VN. This was despite using coating solutions in which FN and VN were at stoichiometrically equivalent concentrations. Based on these data, I conclude that pre-bound FN / IGFBP-5 / IGF-I stimulates SaOS-2 cell migration. Moreover, I hypothesise that the manner in which the combination of matrix protein and growth factors are presented to the cells is critical to elucidating the mechanisms underlying functional cell responses. Importantly, these findings have implications for applications with FN coated

biomaterial constructs, for the induction of cell migration responses into, or onto, the material surfaces.

I also reported in chapter 5 that (in serum-free conditions) FN alone dose dependently facilitated SaOS-2 cell survival, but not proliferation, and that maximal cell survival was observed at around the monolayer saturation limit of 10 µg/mL (Underwood *et al.* 1993; Garcia *et al.* 1999). Furthermore, I reported that pre-binding of both VN and various concentrations of FN to the culture surface induced a dose dependent effect on SaOS-2 cell survival. In addition, this seemed to induce a threshold effect, with SaOS-2 cell proliferation occurring at the highest concentration of FN in the presence of culture surface-saturating levels of VN. Pitt and co-workers have previously reported that multi-layering of FN occurs at high concentrations due to re-orientation of molecular interactions with the adsorbing surface, whereas VN does not re-orientate but does undergo multi-layering (Pitt *et al.* 1987; Pitt *et al.* 1989; Underwood *et al.* 1993). Therefore, I hypothesise that in the present study there were more integrin binding sites available in the presence of VN and the highest dose of FN, thus facilitating a proliferative response. Of course this hypothesis remains to be tested. The finding that standard tissue culture surfaces preferentially bind VN from complex solutions such as serum (Underwood and Bennett 1989) is of interest because subsequent studies have also demonstrated that ECM molecules such as FN and VN undergo concentration dependent conformational changes or multilayering which may in turn affect the biological activity of such coatings (Underwood *et al.* 1993). McFarland *et al.* (1999) determined the amounts of VN and FN bound to tissue culture polystyrene (TCP) surfaces following exposure to culture medium containing FCS. Using ^{125}Iodine labelled VN (^{125}I-VN) or FN (^{125}I-FN) the authors found that the maximal surface density of FN attained in the presence of 10% FCS which had been depleted of both VN and FN was 13 ng / cm^2. Conversely, VN surface density in the presence of FCS was dependent on VN concentration not FCS concentration with 50 µg/mL of VN in a 30% FCS solution resulting in 50 ng/cm^2 of bound VN. This indicated, as mentioned above, that VN preferentially adsorbs to TCP in the presence of FCS. Furthermore, McFarland and co-workers (1999) also found that the coating concentration of purified ^{125}I-VN in PBS was

linearly correlated with the surface density of adsorbed VN with 2 µg/mL of VN resulting in 103 ng/cm^2 of bound VN. Taken together these data clearly indicate that although VN preferentially adsorbs to the TCP surface less VN can adsorb to TCP when in the presence of FCS compared to when in a simple salt solution. This therefore suggests that factors within FCS may impede or slow the adsorption of VN to TCP.

In chapter 3 I reported that pre-bound VN / IGFBP-5 / IGF-I could synergistically enhance SaOS-2 cell proliferation over 72 hr of culture whereas in chapter 5 I showed that pre-bound FN / IGFBP-5 / IGF-I could stimulate enhanced SaOS-2 cell migration in a similar manner to VN. I therefore hypothesised that pre-bound FN / IGFBP-5 / IGF-I could also stimulate SaOS-2 cell proliferative responses. I subsequently showed that pre-bound FN / IGFBP-5 / IGF-I failed to stimulate SaOS-2 cell proliferation to the levels attained for pre-bound VN / IGFBP-5 / IGF-I. This suggests that either, SaOS-2 cells in this system interact with FN and VN, via different mechanisms (ie different integrins) or that FN is unable to retain the same level of IGFBP-5 / IGF-I complex formation as VN and thus delivers less mitogenic signal to the cells. It would therefore be interesting to determine the amount of IGFBP-5 and IGF-I that can be retained by FN or VN, or various combinations thereof, when bound to tissue culture plastic. Similarly, determining the concentration of IGFBP / IGF-I complex retained at the culture surface under standard culture conditions in the presence of 10% FCS would also be of significant interest in order to better understand the intimate microenvironment of cell culture conditions *in vitro*. Moreover, elucidation of the functional concentration of these complexes retained on biomaterial surfaces following exposure to whole human blood (*in vivo*) or serum (*in vitro*) would help determine if adsorption of other molecular species from these complex body fluids could mask the biological activity of the pre-bound complex's studied in this project. Initially, these studies may take the form of epitope mapping investigations similar to those of Underwood *et al.* (1993) or Underwood and Bennett (1989).

As discussed in chapter 5, I reported an interesting preliminary result from a related study in which the effect of serum-free solution phase combinations of VN, FN, bFGF

and EGF on SaOS-2 cell culture expansion were examined. This related study showed that VN / FN / EGF could sustain SaOS-2 cell culture for up to 12 days and the culture appeared generally healthy. Therefore, I decided to include this treatment in a pre-bound format in further studies in order to determine if this format could also elicit functional responses which may be valuable in a tissue engineering context. These studies were aimed at the investigation of the expression levels of select osteoblast markers in response to VN alone, VN / IGFBP-5 / IGF-I, VN / TGF-β_1 and as mentioned VN / FN / EGF. I report in this thesis that after 7 days VN / IGFBP-5 / IGF-I in serum-free conditions induced a change in SaOS-2 cell morphology from a well spread, or polygonal morphology, to a more cobblestone or cuboidal appearance. This type of morphological change is often associated with osteoblast differentiation processes (Aubin 1998). Interestingly, the change in SaOS-2 cell morphology in response to VN / IGFBP-5 / IGF-I occurred while the culture was sub-confluent, compared to similar changes observed in cultures exposed to 10% FCS which had reached confluence prior to a substantial change in cell morphology. This result raised the possibility that VN / IGFBP-5 / IGF-I could stimulate SaOS-2 cells to undergo further differentiation. This was supported by the finding that VN / IGFBP-5 / IGF-I supported the expression and maintenance of ALP gene expression over 7 days and that, in a parallel study performed in our laboratory, resulted in a steady increase in ALP activity over 14 days. However, the ability of VN / IGFBP-5 / IGF-I to facilitate ALP expression may simply represent 'normal' SaOS-2 cell function, rather than a specific differentiation response. SaOS-2 cells are known to express high levels of ALP (Murray *et al.* 1987).

Also of interest was the finding that VN / FN / EGF induced an elongated or fibroblast-like phenotype in confluent cultures of SaOS-2 cells, with few mitotic indices evident after 48 hr and this being maintained until 7 days. In addition, cbfa-1, ALP and Col-I transcript levels in SaOS-2 cells exposed to VN / FN / EGF returned to, or were maintained, at levels equivalent to that determined for un-seeded cells (T_0) after 7 days. Taken together these data suggest that this treatment may have induced these cells into quiescence. This interpretation is supported by my earlier observation that this treatment could support SaOS-2 cells in serum-free culture for up to 12 days, and further by the

findings of others demonstrating EGF / EGFR inhibits osteoblast differentiation processes (Kumegawa *et al.* 1983; Chien *et al.* 2000) and that FN acts as a survival factor for more mature osteoblasts (Globus *et al.* 1998). While the specific mechanisms involved in inducing the observed 'quiescent' state by VN / FN / EGF are at this point unknown, the possibility of reversing this state using other combinations of growth factors, such as BMPs, is intriguing and deserves further investigation. The ability to exert control over cell phenotype and function *in vitro* under serum-free conditions will have significant implications for the design and development of biomaterial constructs in the field of bone tissue engineering.

In conclusion, the data presented in this thesis have confirmed, developed and expanded the findings of others that ECM proteins and growth factors act co-operatively to mediate various epithelial cell functions. Specifically, the data in this thesis extends this concept to include cells of mesenchymal origin, namely osteoblasts and hMSCs. To the best of my knowledge, this thesis presents the first evidence of functional responses in osteoblasts to completely pre-bound VN / IGFBP / IGF-I complexes under serum-free conditions and, as such, contributes to not only the work already performed within our research team but also to the field of bone tissue engineering. This thesis does not attempt to infer *in vivo* biological consequences of the interactions between the matrix proteins and growth factors used in this study; rather it has sought to characterise the functional response of bone forming cells to an artificially constructed ECM under artificial conditions. The initial work performed by other, former members of our laboratory (Hyde 2005; Kricker 2005) focussed on the structural and functional aspects of complexes of VN and various IGF family members in various epithelial cell types *in vitro*. These studies formed the foundation upon which many subsequent studies, including projects involving the serum-free culture of epithelial cell types for corneal replacement, wound healing of burns and diabetic ulcers and the serum-free culture of embryonic stem cells have been pursued. The project reported herein, being the first of its kind in bone-forming cells, has generated substantial data that will form the basis of a number of future studies, especially into the serum-free culture of therapeutically important cells such as hMSCs. In addition the data will inform experiments directed at

examining ECM / growth factor control over bone cell functions in tissue engineered constructs. Thus, it is my belief that the treatments developed and studied within this thesis represent a basic form of an ECM / growth factor, serum-free culture system which might be applied to control cell phenotype and function for future tissue engineering applications.

CHAPTER 7:

APPENDIX

BUFFER RECIPES

7.1 FUNCTIONAL ASSAYS

HEPES Binding Buffer (HBB) pH 7.6

0.1 M HEPES

0.12 M NaCl

5 mM KCl

1.2 mM $MgSO_4 \cdot 7H_2O$

8 mM D-Glucose

7.2 GELATIN ZYMOGRAPHY

5X Loading Buffer pH 6.8

0.05% Bromophenol blue

50% Glycerol

10% SDS

0.5 M Tris base

Running Buffer pH 6.8

25 mM Tris base

246 mM Glycine

0.1% SDS

Incubation Buffer pH 7.6

50mM Tris base

10mM $CaCl_2$

50mM NaCl

Coomasie Blue Stain

0.25% Coomasie Blue

45% Methanol

10% Acetic acid

Destain

40% Methanol

10% Acetic acid

Gelatin Zymograms

(for 4 Gels)	**4% Stacking Gel**	**10% Resolving Gel**
ddH_20	5.4 mL	7.8 mL
1.5 M Tris pH 8.8	-	5.0 mL
0.5 M Tris pH 6.8	2.5 mL	-
Porcine gelatin 10mg/mL	1 mL	2 mL
10% SDS	100 µL	200 µL
40% Bis/Acrylamide	1 mL	5 mL
10% APS	50 µL	100 µL
TEMED	5 µL	10 µL

7.3 SDS-PAGE AND WESTERN BLOT

Running Buffer pH 8.3

25 mM Tris base

200 mM Glycine

0.1% SDS

Transfer Buffer

25 mM Tris base

40 mM Glycine

10% Methanol

Tris Buffered Saline with Tween (TBST)

10 mM Tris base

140 mM NaCl

1% Tween 20

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